

EQUINE MUSCULAR ADAPTATIONS TO
EFFECTIVE USE OF DIETARY FAT
DURING EXERCISE

By

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CHAPTER I

INTRODUCTION

Fat-supplementation in the equine has gained continued popularity primarily as a means of increasing the energy density of feedstuffs without the negative metabolic consequences associated with elevated consumption of highly fermentable carbohydrate meals. Horses are able to maintain body weight and athletic performance while consuming a smaller quantity of feedstuffs. Fat is highly digestible in the equine species and does not appear to affect the digestibility of other nutrients. Furthermore, the long-term feeding of fat to horses has been proven safe. Of particular importance is fat-supplementation in performance horses because of high energy demands and therefore efforts to promote ergogenic effects via fat-supplementation remain controversial. The ability to feed fat as an energy source would theoretically enhance fat oxidation and decrease reliance on carbohydrate oxidation as fat could provide a seemingly endless energy supply whereas carbohydrates as metabolic fuel are quickly diminished in certain athletic efforts. Strategies to promote fat oxidation and therefore spare glycolytic substrates have been well documented in athletic species such as the horse, human, rat and dog. However, each species has its own set of limitations to these strategies. The current study is the first to our knowledge that attempts to relate multiple blood parameters as well as muscle substrate transporter protein expression to the improvement of fat

oxidation by feeding supplemental fat and therefore athletic performance in the equine species.

It is our hypothesis that feeding added dietary fat as compared to a high-starch ration of equal caloric density will promote muscular adaptations that enable greater fat utilization during exercise through the improved use of non-glycogen oxidative substrates, will delay the onset of fatigue during endurance exercise and thus would have a positive impact on endurance by expanding the availability of stored substrates. Our objectives include to: 1) determine if the capacity to transport substrates from the blood stream to the myocyte can be increased 2) evaluate the effect of diet on the basal and exercise-induced substrate concentrations and expression of transport proteins in the skeletal muscle of horses, and 3) determine whether feeding a high-fat diet during training improves the capacity of horses to utilize fat as a substrate to support increased metabolic demands of exercise.

CHAPTER II

REVIEW OF LITERATURE

Fuel for the working muscle

The primary sources of energy for muscle contraction are derived from dietary carbohydrates and fat. While branched chain and other amino acids from protein synthesis can be oxidized in the muscle, their contribution to overall energy expenditure during aerobic exercise is quite small (Jeukendrup et al., 1998a) as their catabolism is considered relatively uneconomic, both fiscally and metabolically (Kronfeld and Downey, 1981; Harris and Kronfeld, 2003). The basic pattern of metabolism of these substrates is set by the nature of the diet (Murray et al., 2006). In general, fuels stored in muscle fibers are phosphocreatine (PCr), glycogen, and triglycerides; fuels from circulation are fatty acids and glucose (Lawrence, 1990; Pösö et al., 2004).

Fat is stored as triglycerides in adipose tissue as the major fuel source for the body and released into the blood as free fatty acids (FFA) through the process of lipolysis (Lawrence, 1990; Wolfe, 1998; Murray et al., 2006). Oxidizable lipid fuels include circulating plasma triacylglycerols (TG) (very-low-density lipoprotein-triacylglycerols - VLDL-TG), albumin-bound long-chain fatty acids (LCFA) in blood plasma, and fatty acids from the triacylglycerol located in the muscle cell (intramuscular triacylglycerols -

IMTG) (Jeukendrup et al., 1998a; Kiens, 2006). Kiens (2006) also includes fatty acids liberated from adipose tissue adhering to muscle cells as a potential energy source.

Other sources of fuel for the exercising muscle derived from fat include ketone bodies such as acetoacetate and betahydroxybutyrate, and glycerol which can be converted into glucose during gluconeogenesis in the liver (Jeukendrup et al., 1998a; Murray et al., 2006).

The storage form of carbohydrate in animals is glycogen which can be stored in the liver or the muscle. Glycogen stored in the muscle can only be used by the muscle itself as muscle cells lack the enzyme glucose 6-phosphatase, which hydrolyzes glucose 6-phosphate into glucose, and thus cannot yield free glucose (Murray et al., 2006). Glucose that is not needed for immediate use or glycogen synthesis may also be used for fat synthesis (NRC, 2007).

The digestive process. While carbohydrates are absorbed as glucose (some fructose and galactose) and enter the portal vein directly, exogenous fats enter systemic circulation much slower (Jeukendrup et al., 1998c). Fats, specifically long-chain fatty acids (LCFA), enter circulation much slower than carbohydrates due to their effect on inhibiting gastric emptying and a slower overall digestive process (Bach and Babayan, 1982). For instance, long chain fatty acids have been reported to enter the blood stream 3 to 4 hours after ingestion by human subjects (Emken, 1994). Nutritional fats include triacylglycerols, phospholipids, and cholesterol; triacylglycerols and potentially phospholipids can contribute in varying degrees during exercise (Jeukendrup et al., 1998c).

Carbohydrate digestion occurs by hydrolysis to liberate oligosaccharides and then to free mono- and disaccharides. Hydrolysis of starch is catalyzed by salivary and pancreatic amylases yielding dextrans, then a mixture of glucose, maltose, and isomaltose. The resulting monosaccharides and others arising from the diet are absorbed in the brush border of the small intestine. Glucose and galactose are absorbed by a sodium-dependent process. Other monosaccharides are absorbed by carrier-mediated diffusion. Fructose and sugar alcohols are only absorbed down their concentration gradient (Murray et al., 2006).

Lingual and gastric lipases initiate the hydrolysis of the triacylglycerol (Murray et al., 2006). In order to split the long-chain triacylglycerol (LCT) into a glycerol and 3 long chain fatty acids, or a monoacylglycerol and 2 LCFA, bile salts from the liver and lipase from the pancreas are needed (Jeukendrup et al., 1998c). Bile acids are synthesized in the liver from cholesterol and stored in the gallbladder of humans and released into the duodenum where they function as a mild detergent to emulsify fat droplets (Borgström, 1974); horses do not have a gall bladder and thus bile salts are secreted directly from the liver (Hintz and Schryver, 1989). In the postprandial state, gallbladder contraction forces the bile salt pool into circulation; bile salts are reabsorbed and re-excreted by the liver as long as digestion is taking place (Borgström, 1974). Likewise, pancreatic lipase is sent to the duodenum through the pancreatic duct, and provides luminal triacylglycerol digestion along with the pancreatic protein, colipase (Murray et al., 2006).

The fatty acids then pass through the intestinal mucosa where, with the help of acyl-CoA synthetase, they are converted to acyl-CoA's which are then incorporated into

LCT, a major component of chylomicrons (Bach and Babayan, 1982). It is important for the LCFA to enter circulation engulfed in the chylomicrons. Chylomicrons, a protein coat to make the fatty acids water soluble, then encapsulate the LCT (Bach and Babayan, 1982). Conversely, short and medium-chain triacylglycerols (SCT, MCT) are not incorporated into chylomicrons, thus they are able to leave the intestine much faster via the portal venous system in the soluble form of fatty acids - short-chain fatty acids and medium-chain fatty acids (SCFA, MCFA) bound to serum albumin (Bach and Babayan, 1982). The LCT now encapsulated by the chylomicrons, is released through the interstitial compartment into the lymphatic system, ultimately draining into systemic circulation (Jeukendrup et al., 1998c; Murray et al., 2006).

Short and MCT are much more rapidly absorbed than LCT as they directly enter systemic circulation through the portal vein (Jeukendrup et al., 1998c; Murray et al., 2006). Compared to LCT, MCT are liquid at room temperature, are more polar (therefore have greater water solubility), and are smaller in molecular size allowing them to be more rapidly digested and absorbed in the small intestine; MCT follow the portal vein and enter the liver directly while LCFA must follow the slow lymphatic system (Bach and Babayan, 1982).

Circulating lipoproteins provide fatty acids. Plasma TG are secreted by the liver and are incorporated into lipoproteins such as chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), intermediate density lipoproteins (IDL), and high density lipoproteins (HDL) and can provide a source of fuel for the working muscle (Havel et al., 1967; Jeukendrup et al., 1998a; Kiens, 2006). In the horse, HDL accounts for 61%, VLDL accounts for 24% and LDL 15% of the total plasma

lipoprotein fraction with no measureable chylomicrons (Hollanders et al., 1986; Watson et al., 1991). Not only do these lipoproteins differ in density, TG and cholesterol content, but they may also fulfill different functions (Jeukendrup et al., 1998a; Murray et al., 2006). Chylomicrons and VLDL may provide energy for metabolism during exercise (Jeukendrup et al., 1998a) as VLDL is the primary lipoprotein responsible for transport of triacylglycerols from the liver to adipose tissue and muscle while HDL transports cholesterol and is involved in VLDL and chylomicron metabolism (Murray et al., 2006). The LDL, IDL, and HDL fractions may not have significant roles in energy metabolism of the exercising muscle (Jeukendrup et al., 1998a). The overall contribution of plasma TG to total fat metabolism during exercise in humans appears to be less than 10% (Havel et al., 1967). However, studies in exercising dogs suggest that circulating TG could represent an important energy substrate for oxidization by the working muscle (Terjung et al., 1982). The TG itself is so energy dense that a significant contribution to energy expenditure for exercise would require only a small fractional amount as Kiens (2006) suggests that circulating TG could provide approximately 10% of necessary fuels during exercise, and may even reach 25% of total energy expenditure after adaptation to a high-fat diet.

As the muscle endothelium is impermeable to lipoproteins, FA must be released from the triacylglycerol forming the core of the VLDL or chylomicrons through the action of lipoprotein lipase (LPL) (Jeukendrup et al., 1998a; Kiens, 2006). Located on the luminal side of the endothelial cells in the capillary bed of most tissues (Ladu et al., 1991; Kiens, 2006; Murray et al., 2006), LPL hydrolyzes some of the TG passing through the capillary wall bed (Jeukendrup et al., 1998a; Kiens, 2006). During fasting and

exercise, LPL activity increased in the skeletal and heart muscle of rats, but decreased in adipose tissue (Ladu et al., 1991). Furthermore, as there is a positive correlation between LPL activity in skeletal muscle and capillarization, and since endurance training increases capillarization (Kiens and Lithell, 1989), LPL activity is significantly increased after training (Kiens and Lithell, 1989; Kiens et al., 2004) as well as stimulated during acute exercise (Ladu et al., 1991). Horses are unique in relation to other athletic species in that there is an increase in circulating TG during exercise, and this increase correlated with exercise intensity and lipolysis activity (Pösö et al., 1989).

Nutritional status also increases skeletal muscle LPL activity and muscle TG storage (m. vastus lateralis) after a high-fat diet as compared to a high-carbohydrate diet in physically active men (Kiens et al., 1987). Studies in horses found a linear relationship between fat intake and plasma LPL activity (Geelen et al., 2001b) regardless of the type (saturated vs. unsaturated) of fat fed (Hallebeek and Beynen, 2002). It was concluded that for every 1 g/kg dry matter increase in fat intake, LPL activity increases by 0.98 $\mu\text{mol fatty acid released}\cdot\text{mL}^{-1}\cdot\text{h}^{-1}$, but there was not a statistically significant effect on plasma TG concentrations in horses (Geelen et al., 2001b). Marchello et al. (2000) found that the VLDL but not the LDL fraction of TG increased in horses fed a high-fat diet, indicating an increase in VLDL-TG clearance from the circulation presumably due to increased LPL activity which could also be due to an increase in liver-produced digestive products. Peterson et al., (1990) suggests that, due to the strong correlation of LPL activity to increased FFA levels in plasma, the major rate-limiting factor in plasma TG turnover may be the capacity of the tissues to metabolize liberated fatty acids rather than the ability of LPL to release the fatty acids from the TG.

The contribution of VLDL-TG to energy expenditure during exercise may not be negligible even though attempts to measure it directly report otherwise (Jeukendrup et al., 1998a). The magnitude of the contribution can depend on diet, exercise intensity, and duration (Kiens, 2006). Likewise, arterial LCFA concentration depends upon the pre-exercise diet, the amount of time since last meal, and carbohydrate consumption (Kiens, 2006).

Triacylglycerols stored in muscle. Triacylglycerol pools in the muscle are made up of LCFA from circulation, albumin-bound FA, and from VLDL-TG that are degraded by LPL (Kiens, 2006). *Intramuscular* triacylglycerols are stored in the muscle cell, primarily as lipid droplets adjacent to the mitochondria (Hoppeler, 1986; Kiens, 2006) in comparison to the larger amount of *intermuscular* TG which is triacylglycerol from adipocytes that is interlaced between muscle fibers (Jeukendrup et al., 1998a; Kiens, 2006). Skeletal muscle TG content is dependent upon nutrition, muscle fiber type and physical exercise.

A high-fat diet increases TG content in skeletal muscle, while a carbohydrate-rich diet has the opposite effect and the dietary influence may be related to the activity of muscle LPL (Kiens, 2006). For instance, as the amount of dietary fat increases, so does the LPL activity in skeletal muscle, leading to increased concentration of IMTG (Kiens et al., 1987). Conversely, as carbohydrate amounts in the diet are increased, generally the higher the plasma insulin concentration which decreases muscle LPL activity (Kiens et al., 1987). Furthermore, in a situation where training is combined with a high-fat diet, the increase in IMTG is positively correlated with an increase in muscle LPL activity (Kiens, 2006).

The concentration of IMTG appears to be fiber-type specific. Essén (1977) concluded that in man, all fiber types have similar concentrations of glycogen, but that high oxidative muscle fibers tend to have a higher concentration of TG as compared to high glycolytic fibers, which are less dependent upon fat oxidation. A similar trend was found in rat muscle in which highly oxidative muscle fibers contained a higher concentration of TG (Spriet et al., 1986). Conditioning in equine subjects likewise brings about an increase in intramuscular lipid droplets (Tyler et al., 1998; Serrano et al., 2000).

Conditioned individuals have a greater amount of intracellular lipid deposits (Hoppeler, 1986). Conditioned subjects are less reliant on plasma TG stores suggesting that they are able to oxidize more muscle TG overall (Martin et al., 1993), which may imply that intramuscular TG stores are of significance with regard to energy provision. It must be noted, however, that there must be an ample amount of fat in the diet in order for an increase in IMTG with conditioning to occur (Kiens, 2006).

Muscle triacylglycerol is an important substrate for working muscle; however, as there exists great difficulty in precise quantification of its contribution to overall energy expenditure, it is not fully understood just how important.

Glycerol and ketone bodies. Other fat metabolites such as glycerol and ketone bodies can also be used as fuel during exercise. Glycerol is released from adipose tissue after hydrolysis of TG and transported to the vascular space. In the fed state, glycerol can be used for reesterification of free fatty acids to TG in the liver or adipose tissue while in the fasted state, glycerol released from adipose tissue is then transported by the blood to the liver or kidney where it serves as a gluconeogenic precursor for glucose (Murray et al., 2006). However, since the glycerol is not rapidly cleared from the blood and cannot

be directly used by the working muscle, glycerol is thought to contribute very little to energy expenditure during exercise (Murray et al., 1991).

Ketone bodies such as acetoacetate, beta-hydroxybutyrate, and acetone are products of incomplete fatty acid oxidation, arising from partial degradation of LCFA liberated from adipose tissue during times of fasting or mediated by catecholamines; lipogenesis in adipose tissue predominates in the fed state (Fukao et al., 2004). During high rates of fatty acid oxidation, the efficiency of the Krebs's cycle is reduced and excess acetyl-CoA is shuttled to ketone body production (Hartman et al., 2007). Fasting ketosis occurs after progressive depletion of postprandial nutrients and subsequently, tissue glycogen depletion (Fukao et al., 2004). Glucose and insulin suppress ketogenesis; ketogenesis requires glucopenia, or hypoglycaemic conditions (Fukao et al., 2004). Under normal circumstances, only the liver can produce ketones from the pool of hepatic intramitochondrial acetyl CoA, but most tissues, including skeletal muscle, can oxidize them (Fukao et al., 2004). Ketone body concentration is normally very low $50 - 150 \text{ mmol} \cdot \text{l}^{-1}$ in humans, but may increase rapidly after fasting or during prolonged exercise in humans and rats (Dohm et al., 1983; Dohm et al., 1986; Brouns et al., 1989). As ketone bodies are very rapidly cleared from circulation and metabolized by extrahepatic tissues (VanItallie and Nufert, 2003), the production of ketones is extremely important as the brain can only use ketone bodies and glucose as fuel; therefore, ketone bodies can serve as an alternative fuel for glucose during times of fasting in both the brain and muscle cells (Jeukendrup et al., 1998a). Conditioning appears to increase the capacity of the muscle to use ketones as a substrate for energy (VanItallie and Nufert, 2003) and exercise in itself promotes ketogenesis (Robinson and Williamson, 1980). Many factors

can contribute to ketone body utilization during exercise, such as species, diet, fasting state, and the exercise itself. Humans are more susceptible to ketosis, as compared to canines, which are better adapted to high fat diets and more resistant to hypoglycaemia, (Hammel et al., 1977; de Bruijne et al., 1981; de Bruijne and van den Brom, 1986). Rose and Sampson (1982) concluded that the ketone pathway in the horse is relatively unimportant, as signified by only a small increase in both circulating concentrations of non-esterified fatty acids (NEFA) suggesting that mobilization exceeded utilization, and hepatic ketoacid production after endurance exercise and food deprivation. This finding is potentially due to the ability of the liver to maintain glycogenolysis and therefore the carbohydrate supply for the citric acid cycle (Rose and Sampson, 1982). Nonetheless, more strenuous exercise such as three-day event competition significantly decreases ketone concentration post-exercise in horses, perhaps shedding light on the differences in NEFA metabolism between the horse and man (Rose et al., 1980). The horse seems to respond to increased fat breakdown by production of FFA, not ketoacids (Naylor et al., 1980).

Why fat is the substrate of choice: fat versus carbohydrate

Many biochemical and physical properties of fats distinguish them from carbohydrates. For instance, fat contains more than twice as much energy as carbohydrate: $38 \text{ kJ} \cdot \text{g}^{-1}$ ($9 \text{ kcal} \cdot \text{g}^{-1}$) for fats versus $18 \text{ kJ} \cdot \text{g}^{-1}$ ($4 \text{ kcal} \cdot \text{g}^{-1}$) for carbohydrates (Jeukendrup et al., 1998a). Murray et al. (2006) reports similar numbers 37 kJ/g for fat and 16 kJ/g for carbohydrates (see Table 1). In addition, while carbohydrates are stored in the presence of water, fat is stored anhydrous. Therefore, fat

is considered a more efficient fuel per unit of weight; our weight would double if all of our fat was replaced by an energetically equal amount of carbohydrates (Jeukendrup et al., 1998a).

Table 1. Carbohydrates versus fats (Adapted from Murray et al., 2006)

	Energy Yield kJ/g	O ₂ Consumed (L/g)	CO ₂ Produced (L/g)	RQ = CO ₂ /O ₂
Carbohydrate	16	0.829	0.829	1.00
Fat	37	2.016	1.427	0.71

Fat oxidation has the capability to provide more energy per unit of the substrate, as more ATP is produced per molecule of fat than carbohydrate: 38 mol of ATP from aerobic glucose oxidation compared to 106 mol of ATP per mole of palmitate for comparison (Murray et al., 2006). Nonetheless, oxidation of fat does require more than twice the amount of oxygen than that of carbohydrate; therefore, *per unit of time*, more ATP can be derived from glucose (Murray et al., 2006).

High-energy phosphates act as the “energy currency” of the cell and are donated by ATP to form compounds such as phosphoenolpyruvate, creatine phosphate, glucose 1 & 6-phosphate, and glycerol 3-phosphate among others (Murray et al., 2006). Further, there are three primary sources of high-energy phosphates that take part in energy conservation or energy capture: 1) Oxidative phosphorylation; 2) Glycolysis; 3) Citric acid cycle (Murray et al., 2006). Oxidative phosphorylation produces the greatest quantity of high-energy phosphates from respiratory chain oxidation using molecular O₂ within the mitochondria. Glycolysis forms two high-energy phosphates from the

formation of lactate from one molecule of glucose and one high-energy phosphate is generated in the succinyl thiokinase step of the citric acid cycle (Murray et al., 2006).

Gollick (1985) concludes that the amount of ATP present in a muscle cell at any given time is so small that it can rapidly be depleted and in order for muscular activity to continue, resynthesis of ATP must continually take place by one or more of the following: 1) transfer of HEP from creatine phosphate, 2) the myokinase-catalyzed reaction: $2\text{ADP} \rightarrow \text{ATP} + \text{AMP}$, 3) degradation of carbohydrate to lactate, and 4) the terminal oxidation of fats and carbohydrates to CO_2 and H_2O . Each method can be important depending on type and duration of contractile patterns involved.

Fuel storage, fat and carbohydrate. In comparison to carbohydrate stores, fat stores are very large. In theory, fat stores could provide energy for days whereas carbohydrate stores could become depleted within 60 to 90 minutes in the human athlete; approximately 500g of glycogen could fuel 95 minutes of running for an elite marathon runner (Jeukendrup et al., 1998a).

Fuel derived from carbohydrates is primarily stored in skeletal muscle as glycogen, although liver glycogen and extracellular glucose can also contribute (Murray et al., 2006). Exogenous carbohydrate sources can also provide glucose for oxidative processes in the muscle after absorption in the gut and upon entering circulation. As muscle cells lack the enzyme glucose 6-phosphatase, they cannot export glucose to be used by other cells; muscle glycogen provides glucose only for glycolysis within the muscle itself to be used directly as fuel. Liver glycogen functions as storage and exportation of glucose to extrahepatic tissues and requires transport first by the blood before it can be taken up by these tissues to be used as fuel (Murray et al., 2006). Other

Gluconeogenic fuels include lactic acid, glycerol, and amino acids which can be converted into glucose in the liver and indirectly used as energy (Jeukendrup et al., 1998a).

Carbohydrate stores in man are small, as muscle mass in a normal human can contain only 400-500 g of glucose or 6,500 kJ of energy (Essén, 1977) although conditioning can increase these stores (Jeukendrup et al., 1998a). In contrast, equine muscle has a very high capacity to store glycogen, up to 600–650 mmol/kg dry muscle in conditioned individuals; similar values for humans can only be obtained after successful carbohydrate loading (Pösö et al., 2004). Liver glycogen stores in the 80kg man represent 80-100 grams and plasma substrates such as glucose and lactate are about 10-20g (Jeukendrup et al., 1998a; Murray et al., 2006). Human carbohydrate stores can total approximately 8,000 kJ (2,000 kcal) when expressed in terms of energy (Jeukendrup et al., 1998a).

The largest quantity of fat is stored in adipose tissue and the storage of fat is dynamic meaning that in conditions of negative energy balance, the size of fat cells decrease, while in case of positive energy balance, excess fat is converted to triacylglycerols and fat cells hypertrophy as a result (Jeukendrup et al., 1998a). In addition to adipose tissue, the IMTG content in man can vary greatly, but is normally around 5-15 mmol/kg wet weight totaling 200-300 g or 10,000 kJ (Essén, 1977). Furthermore, the more highly oxidative type I muscle fibers (207 mmole/kg d.w.) tend to have 2-3 times the triacylglycerol content than type II fibers (74 mmole/kg d.w.) whereas glycogen content between fiber types was very similar (Essén, 1977).

The contribution of fat or carbohydrate to exercise. Traditionally, the contributions of fat or carbohydrate in exercise can be measured by the respiratory exchange ratio (RER) which can also be called the respiratory quotient (R or RQ). This system is based upon the fact that fat and carbohydrates produce different amounts of carbon dioxide (CO₂) and require different amounts of oxygen (O₂) when they are oxidized (Jeukendrup et al., 1998a). Therefore, it is possible to obtain substrate utilization information by using VO₂ and VCO₂ measurements in expiratory gases: $R = VCO_2 / VO_2$. An R-value of 1.0 indicates that carbohydrate is the primary fuel source oxidized whereas an R-value of 0.69 – 0.73 indicates that fat is providing a majority of the fuel (Lawrence, 1990; Jeukendrup et al., 1998a; Murray et al., 2006). Contributions to RER change according to diet, exercise intensity, physical fitness, fiber type recruitment pattern and duration (Essén, 1977; Lawrence, 1990). Other methods of investigation of the kinetics of substrate utilization and mobilization now exist such as radioactive isotope tracers and tracer dilution techniques along with the reintroduction of the muscle biopsy technique in the late sixties by Bergström and Hultman (Romijn et al., 1993; Jeukendrup et al., 1998a).

The response of lipid metabolism to exercise is complex, it can depend on exercise intensity, duration, and level of conditioning. At prolonged low intensity exercise, such as 40% VO_{2max}, glycolysis is not stimulated and fatty acids are available to meet energy demands (Wolfe, 1998). At a higher intensity of exercise (85% VO_{2max}), there is an increased amount of pyruvate into the TCA cycle for oxidation due to the stimulation of glycogen breakdown and glycolysis; however, FA oxidation is inhibited by limiting transport into mitochondria (Wolfe, 1998).

Regulation of substrate utilization – hormonal regulation

Changes in hormone concentrations can explain changes in gluconeogenesis, lipolysis and ketogenesis during exercise (Jeukendrup et al., 1998b). Insulin and glucagon control the formation and utilization of TG and glycogen as well as the extent to which glucose is oxidized by tissues (Murray et al., 2006). Hormones primarily effect the mobilization of FA (lipolysis). In general, catecholamines increase lipolysis while insulin inhibits lipolysis. As insulin levels decrease, levels of glucagon, epinephrine, norepinephrine, growth hormone (GH), and cortisol all increase (Jeukendrup et al., 1998b) resulting in an increase in hormone sensitive lipase and subsequently an increase in circulating FFA (McKeever and Gordon, 2004). On the other hand, an increase in insulin will inhibit the release of FFA from adipose tissue by inhibiting the activity of hormone sensitive lipase resulting in a decline in circulating plasma free fatty acids (Murray et al., 2006). In addition, insulin enhances lipogenesis, in which glucose is converted into fatty acids, and the synthesis of acylglycerol (Murray et al., 2006). Since insulin facilitates glucose uptake by the cells, it is a promoter of glycogenesis and an inhibitor of gluconeogenesis; hence, insulin is vital to the recovery phase of exercise for glycogen repletion (McKeever and Gordon, 2004). As expected, glucagon acts in opposition to insulin in that it inhibits glycogenesis and stimulates gluconeogenesis – important for the maintenance of glucose concentrations during exercise (McKeever and Gordon, 2004). In simple terms, insulin is an indicator of blood sugar level in the body; thus high insulin levels indicate the “fed” state. Therefore, the plasma concentration of these hormones is primarily dependent upon plasma glucose concentration as well as the

ability of insulin to enhance the uptake of glucose into adipose cells via the GLUT4 transporter (Murray et al., 2006). It is also noted that plasma catecholamines and sympathetic neural activity rise exponentially with increasing exercise intensity (Jeukendrup et al., 1998b) in a curvilinear fashion and are highly correlated with plasma lactate concentrations (McKeever and Gordon, 2004). In horses, humans, and other species, acute exercise suppresses insulin with rising catecholamine concentrations; however this suppression appears to have a limit of 50% $\text{VO}_{2\text{ max}}$ (Thornton, 1985). The effect of plasma catecholamine concentrations on lipolysis and fat oxidation during exercise has not been thoroughly described, so other factors may play a major role in exercise at moderate to high intensities (Jeukendrup et al., 1998b) such as adipose tissue blood flow (Bülow, 1982), plasma insulin concentration or time of feeding (Duren et al., 1999), plasma lactate concentration, and increased glycolytic flux (Shaw et al., 1975).

Two hormones that deal with energy homeostasis, leptin and adiponectin, are both secreted by adipose tissue and are proportional to fat mass in horses (Kearns et al., 2006; McKeever and Gordon, 2004). Leptin is positively correlated with percent fat mass while adiponectin is negatively correlated (McKeever and Gordon, 2004; Kearns et al., 2006).

Leptin acts as an indicator of energy sufficiency rather than excess (Murray et al., 2006) and increases with adiposity (Kearns et al., 2006). In horses, plasma leptin is positively correlated with percent fat mass (Kearns et al., 2006) and body condition score (McKeever and Gordon, 2004). In humans, leptin is likewise positively correlated to fat mass and % body fat (Weltman et al., 2000). High levels of leptin increase energy expenditure while decreasing food intake (McKeever and Gordon, 2004). Plasma leptin

concentrations have been found not to change during or immediately after exercise in humans (Essig et al., 2000) and horses (Gordon et al., 2007b). However, short-term, high intensity exercise in horses decreases plasma leptin concentration post-exercise, signaling alterations in energy intake in order to maintain energy balance (Gordon et al., 2007b). At the same time, long-term exercise in humans can also decrease leptin concentrations due to an increase in the energy deficit (McKeever and Gordon, 2004). The duration in which this takes place varies between the human and equine species.

Leptin has been found to be a potent stimulator of lipolysis and fatty acid oxidation in adipocytes and other cell types. In vitro studies have shown that the influence of leptin can change the fuel source from which ATP is generated – switching preference from carbohydrates (glucose) to lipid (fatty acids); therefore, leptin is a regulator of cellular TG content (Reidy and Weber, 2000). In short, a negative energy balance can result when leptin levels increase and food consumption decreases in combination with a relatively high energy expenditure. An inhibition on insulin's lipogenic effect in adipose as well as skeletal muscle can also occur (Reidy and Weber, 2000), but not insulin's effect on glycogen synthesis, glucose oxidation or lactate production in non-adipocytes. In summary, leptin decreases food intake and partitions metabolic fuel towards utilization and away from storage (Muoio et al., 1997).

Adiponectin concentrations have also been found to not be altered with respect to exercise in both horses and humans (Gordon et al., 2007b). It is suggested, in humans at least, that repeated exercise bouts may be needed to see changes on plasma adiponectin concentrations due to exercise (Gordon et al., 2007b).

Adiponectin has been shown to have potent effects on carbohydrate and lipid metabolism in skeletal muscle (Karbowska and Kochan, 2006). Adiponectin has been linked to the up-regulation of several genes involved in muscle lipid metabolism, including FAT/CD36 (Karbowska and Kochan, 2006). The adiponectin-dependent increase in muscle fatty acid oxidation perhaps is due to the decrease in malonyl-CoA concentration caused by an adiponectin-dependent increase in the phosphorylation of AMP-activated protein kinase (AMPK) [*which phosphorylates (inhibits) activity of acetyl-CoA carboxylase (ACC) and subsequently decreases malonyl-CoA concentration which is an inhibitor of carnitine palmitoyl transferase 1 (CPT1)*] (Karbowska and Kochan, 2006). Globular adiponectin can also increase glucose uptake into the muscle via enhanced translocation of glucose transporter 4 (GLUT4) but reduces glycogen synthesis in rat skeletal muscle (Ceddia et al., 2005).

Regulation of substrate utilization – malonyl-CoA

It has been suggested that the action of the enzyme carnitine palmitoyltransferase 1 (CPT1), located on the outer mitochondrial membrane, is another possible rate-limiting step for transport of LCFA into the mitochondria and is an important regulatory site of fatty acid oxidation (Jeukendrup et al., 1998b; Kiens, 2006). Carnitine palmitoyltransferase 1 is very sensitive to changes in concentration of malonyl-CoA which can act as a potent inhibitor (Saggerson, 2008). Studies in humans and rodents suggest a close relationship between an increase in malonyl-CoA correlating with a decrease in fatty acid oxidation and vice versa.

Malonyl-CoA is formed from acetyl CoA catalyzed by the enzyme acetyl-CoA carboxylase (ACC) (Kiens, 2006; Saggerson, 2008). Regulation of ACC can include allosteric activation by cytosolic citrate concentrations (Roepstorff et al., 2005; Saggerson, 2008) or AMPK (5'-AMP-activated protein kinase) activity which increases with exercise in human skeletal muscle and can phosphorylate and inhibit ACC; therefore, decreasing malonyl-CoA concentration (Kiens, 2006). On the other hand, glucose and insulin may stimulate ACC, allowing malonyl-CoA to accumulate which may inhibit carnitine dependent LCFA transport across the mitochondria (Jeukendrup et al., 1998b; Saggerson, 2008). When skeletal muscle is supplied with a surplus of fuels such as glucose, malonyl-CoA concentration increases. High insulin and glucose availability at rest has been shown to elevate cytosolic citrate concentration causing an increase in muscle malonyl-CoA concentration at rest in both rodents (Saha et al., 1997) and humans signifying the likelihood that this is the mechanism that inhibits fatty acid oxidation by high glucose availability in resting humans (Båvenholm et al., 2000). Regardless of muscle glycogen levels, the increase in fat oxidation at the onset of exercise is due to the decrease in malonyl-CoA concentration brought about by AMPK activation and thus ACC inhibition by phosphorylation (Roepstorff et al., 2005).

Even so, malonyl-CoA concentrations have yet to be linked directly to changes in fat oxidation during exercise in either human or rat muscle (Jeukendrup et al., 1998b). It is highly likely that mechanisms other than changes in malonyl-CoA concentration, ACC activity or AMPK activity are involved in regulation of lipid utilization during exercise (Kiens, 2006). Also, with high concentrations of muscle glycogen present, fat oxidation

may be limited during exercise because of the availability of free carnitine to CPT1 due to its increased use for acetylcarnitine formation (Roepstorff et al., 2005).

Regulation of substrate utilization – carnitine

Carnitine is produced endogenously in hepatic tissue, liver, and brain. Exogenous sources include those from the diet primarily animal sources with a lesser amount present in grains, fruits and vegetables (Steiber et al., 2004). L-carnitine is not produced in the muscle, so muscle carnitine concentration is dependent upon synthesis and diet (Jeukendrup et al., 1998b). Carnitine is synthesized from the amino acids lysine and methionine and biosynthesis in humans is determined by availability of trimethyllysine from tissue proteins (Steiber et al., 2004). Ingestion of synthetic L-carnitine increases plasma carnitine, but has no effect on muscle carnitine concentration in humans (Soop et al., 1988; Lee et al., 2007) or horses (Harris et al., 1995; Foster et al., 1988) as the uptake of L-carnitine must go against a very large concentration gradient (plasma carnitine concentration is about 100X less than that of muscle carnitine concentration) (Jeukendrup et al., 1998b) and low intestinal absorption (Foster et al., 1988). Therefore, fatty acid oxidation appears to be uninfluenced by carnitine supplementation (Ceretelli and Marconi, 1990). Carnitine is critical for β -oxidation as it is a substrate for CPT1 and is thereby required for transport of fatty acyl CoA across the inner mitochondrial membrane and into the matrix (Steiber et al., 2004). In turn, carnitine could play a valuable role in the regulation of the oxidation of lipid substrates. Studies have shown that fat oxidation at rest is not limited by carnitine (Roepstorff et al., 2005) but carnitine could play a role in lipid oxidation during exercise (Kiens, 2006). As exercise induces an increase in

glycogenolysis and glycolysis, acetyl CoA is generated by the mitochondria in response to the action of the PDH complex, carnitine can then be acetylated by acetyl CoA (Kiens, 2006). This in turn makes it act as a sink for accumulating acetyl groups under conditions where rate of acetyl CoA formation from pyruvate exceeds rate of utilization by TCA cycle (Kiens, 2006). Therefore, at increasing exercise intensities the amount of acetylcarnitine increases in both horses and humans, ultimately limiting the amount of free carnitine to act as a substrate for CPT1 and thus slowing the rate of fat oxidation because of diminished supply of LCFA-CoA into β -oxidation (Foster and Harris, 1987; Roepstorff et al., 2005; Kiens, 2006). Further, low pre-exercise muscle glycogen concentrations, brought about by a low carbohydrate diet, provide few acetyl groups to acetylate carnitine. Therefore, the concentration of free carnitine and rate of fat oxidation is higher during submaximal exercise than when muscle glycogen levels are high (high-carbohydrate diet) and free carnitine concentration in skeletal muscle is low (Roepstorff et al., 2005). It is important to note that while total carnitine concentration in skeletal muscle is unaffected by exercise in horses (Foster and Harris, 1987) and humans, lipid oxidation may very well be related to availability of free carnitine (Kiens, 2006).

Other regulators of substrate utilization

Muscle lactate accumulation and therefore a decline in pH can also contribute to reduced fat oxidation. In vitro studies performed on rat and human skeletal muscle when muscle pH declined from 7.1 to 6.8 produced a significant decreased of 34-40% in CPT1 activity both in sarcolemmal and interfibrillarly located mitochondria (Bezaire et al., 2004). The link between CPT1 and pH is reasonable given that at low intensity,

submaximal exercise, the rise in muscle lactate and subsequent decline in pH is quite small, but at high intensity exercise where muscle lactate concentration is high, there could be a more drastic decline in pH (Sahlin, 1978). Therefore, this could decrease lipid oxidation as an exercise-induced decrease in CPT1 activity is the likely contributing mechanism to the reduction in lipid oxidation at high intensities (Kiens, 2006).

Another regulator of substrate utilization could be the inhibition of β -ketoacyl-CoA thiolase, the enzyme responsible for catalyzing the final reaction in β -oxidation, by the accumulation of acetyl CoA, causing a reduction of β -oxidation (Kiens, 2006).

The intensity of exercise determines the mechanism of fatigue in equine athletes. For example, acidification is the primary cause of fatigue in high intensity exercise, while depletion of energy stores will largely constitute the mechanism of fatigue in low-intensity exercise situations (Pösö et al., 2004). Accumulation of lactate and ultimate decline in pH occurs once exercise has exceeded the anaerobic threshold (Pösö et al., 2004) and accumulation in the blood and muscle positively correlates with the amount of glycogen utilized and oxidative capacity of the muscle (Valberg et al, 1985).

The extent of lactate accumulation as well as its removal is due in no small part to the activity of monocarboxylate transporters (Green et al., 2002). Monocarboxylate transporters 1 and 4 (MCT1 and MCT4) are both found in skeletal muscle type 2A muscle fibers (Kobayashi, 2004). Monocarboxylate transporter 1 concentration is greatest on the plasma membranes of highly oxidative muscle fibers (Type 1), is highly related to the oxidative capacities of skeletal muscles, and functions to take up lactate into the muscle cell where it can be metabolized (McCullagh et al., 1996; Kobayashi, 2004). Monocarboxylate transporter 4 is found in muscle fibers with greater anaerobic

glycolytic capacity and is localized on the plasma membranes of all Type 2b fibers and is involved in the efflux of lactic acid out of muscle cells (Kobayashi, 2004). In equine muscle, the isoform profile of MCT4 was found to be similar to that of man and tended to increase with age (Koho et al., 2006). McCullage et al. (1996) found that MCT1 was highly related to the oxidative capacities of skeletal muscle and their capacity to take up lactate into the cell to be metabolized. Further, MCT1 was highly related to lactate dehydrogenase (LDH) enzymes which are necessary for the conversion of lactate to pyruvate thus enabling oxidation of the substrate (McCullagh et al., 1996). Monocarboxylate transporter 1 has also been found to transport ketones β -hydroxybutyrate and acetoacetate across the blood-brain barrier and its expression in the rat brain was increased 6-fold in response to a ketogenic diet (Leino et al., 2001).

Limitations of fat oxidation

Carbohydrates in many cases continue to be the dominant substrate in physical activity because, while fat stores are relatively high, the capacity to oxidize fat substrates is slow, if not limited. Because the metabolic pathways are identical for metabolism of fat and carbohydrate beyond the formation of acetyl-CoA, the rate limiting step for the oxidation of fatty acids must be prior to the TCA cycle. Nonetheless, any of the following steps may be rate-limiting with respect to fatty acid oxidation.

1. Mobilization of FA from adipose tissue
2. Removal of FA from adipose tissue
3. Transportation of FA to the muscle cell by the blood
4. Fatty acids are taken up by the muscle cell

5. Mobilization of FA from intramuscular triacylglycerol pools
6. Transportation of FA into the mitochondria of the muscle cell
7. Oxidation of fatty acids occurs in the mitochondria

1) Mobilization of fatty acids from adipose tissue. Most tissues contain only small amounts of fat and the ability to use those fats is small compared to fatty acid turnover in exercise; tissues therefore depend on a continuous supply of fatty acids from the diet as well as adipose tissue (Jeukendrup et al., 1998a). The rate of lipolysis, the rate of esterification of fatty acids within the adipocyte, and the transport of fatty acids from the adipose tissue to the blood all contribute to the rate of mobilization of fatty acids from adipose tissue (Jeukendrup et al., 1998a). Fatty acids are constantly mobilized by a process called lipolysis (hydrolysis of triacylglycerols) in adipose tissue, liver, and muscle, with most lipolysis occurring in adipose tissue as that is where a majority of the body's TG stores are located. Therefore, lipolysis in adipose tissue controls the release of FA into plasma where they circulate as FFA bound to albumin (Coppack et al., 1994; Murray et al., 2006).

Lipolysis begins with the transfer of triacylglycerols from the main lipid droplet to the site of enzymatic cleavage in the cytoplasm of the adipocyte by the enzyme hormone-sensitive lipase (HSL), forming a monoacylglycerol and two fatty acids. The fatty acids diffuse into circulation while the remaining monoacylglycerol is split by the enzyme monoacylglycerol lipase (MGL) into glycerol and another fatty acid; this fatty acid also diffuses into circulation (Jeukendrup et al., 1998a). Glycerol is a small water soluble molecule that can more easily diffuse through the cell membrane and into the blood than

glucose; it may be used for reesterification of TG in adipose tissue or the liver or may be a substrate for gluconeogenesis in the liver (Murray et al., 2006). Fatty acids can be used as fuel in most tissue except the brain and erythrocytes (Murray et al., 2006) or reesterified to form new triacylglycerols in the adipocyte in a process called triacylglycerol-fatty acid cycling (Jeukendrup et al., 1998a).

As HSL is subject to hormonal regulation by insulin and catecholamines primarily, it can be considered a rate-limiting step in the process of fatty acid mobilization from adipose tissue (Hales et al., 1978; Coppack et al., 1994; Murray et al., 2006). Hormone sensitive lipase is present in both an active and an inactive form, subject to adrenergic stimulation leading to phosphorylation of the inactive hormone, thereby activating it; insulin will stimulate a phosphodiesterase leading to decreased cAMP levels and thus decreased HSL activation (Hales et al., 1978). Insulin is said to be the most potent antilipolytic hormone and has been extensively studied (Hales et al., 1978; Coppack et al., 1994; Murray et al., 2006). Ketone bodies acetoacetate and β -hydroxybutyrate are also said to be inhibitors of lipolysis, although the exact mechanism is not clear (Coppack et al., 1994).

Other hormones act to increase the rate lipolysis in adipose tissue thereby accelerating the release of FA and thus increasing plasma FFA concentration. Circulating concentrations of epinephrine, norepinephrine, glucagon, adrenocorticotrophic hormone (ACTH), α - and β -melanocyte-stimulating hormones (MSH), thyroid stimulating hormone (TSH), growth hormone (GH) and vasopressin among others provide stimulation to adrenergic receptors and activate HSL, stimulating lipolysis (Coppack et al., 1994; Murray et al., 2006).

In humans at least, catecholamines have a dual effect on lipolysis, while alpha-adrenergic inhibitory mechanisms modulate lipolysis at rest and beta-adrenergic stimulating effects occur during exercise (Wahrenberg et al., 1987). At low to moderate intensity exercise, lipolysis would increase due to the sensitivity of beta-adrenergic receptors for increasing catecholamine concentration in adipose tissue (Wahrenberg et al., 1987). At high intensity exercise, greater than 80% VO_{2max} , high circulating levels of epinephrine along with increased glycolytic flux and increased $[LA]_{plasma}$, will tend to decrease lipolysis and therefore increases reesterification in adipose tissue (Jeukendrup et al., 1998a).

Exercise onset in horses increases the concentration of circulating catecholamines therefore decreasing plasma insulin concentration during exercise due to inhibition effect from epinephrine and somewhat from norepinephrine upon pancreatic insulin release (Pösö et al., 2004). The overall effect is to increase lipolysis since increased concentration of plasma insulin would inhibit lipolysis.

2) *Removal of fatty acids from adipose tissue: reesterification vs. release.* The rate of lipolysis actually exceeds the need for FA in oxidative processes; therefore, FA mobilization is regulated by reesterification (Wolfe 1998). Reesterification can occur within the adipocyte “intracellular reesterification” or the fatty acid can be released and reesterified in some other tissue “extracellular reesterification” (Jeukendrup et al., 1998a). Remember that glycerol cannot be incorporated into triacylglycerols due to the absence of glycerol kinase. Glycerol kinase is indirectly derived from glucose in the adipose tissue and is required for reesterification; it is used in adipose tissue to convert

glycerol to glycerol-3-phosphate (G-3-P), the back bone of the triacylglycerol molecule (Jeukendrup et al., 1998a).

The stimulation of lipolysis is very rapid after the onset of exercise and during low intensity exercise lipolysis will continue to increase; however, lipolysis cannot increase proportionally as exercise intensity increases and at approximately 65% of VO_{2max} , fatty acid oxidation becomes equal to the total amount of fatty acids available (Wolfe, 1998). Shaw et al. (1975) also concludes that the most pronounced suppression of R_a FFA (rate of appearance of FFA) and drop in the R_a FFA/ R_a Glycogen ratio occurs at the onset of exercise and subsequently rises as the stimulus for lipolysis becomes increasingly stronger due to the decrease of plasma glucose and increase of plasma norepinephrine. Remembering that adipose tissue blood flow is increased during exercise, R_a of FA in plasma are also increased as a result; therefore, the rate of reesterification is also dependent upon the ability of the plasma to transport the released FA (i.e. the number of free albumin binding sites) and availability of glucose to produce G-3-P (Jeukendrup et al., 1998a). Moreover, lactate has been demonstrated to increase the rate of fatty acid reesterification by as much as 35–40 % at heavier exercise intensities in dogs when plasma lactate levels rose from 6.5 to 18.6 mg/100ml (Shaw et al., 1975).

The rate of removal of fatty acids from adipose tissue could be dependent upon the plasma albumin concentration, arterial fatty acid/albumin ratio, and blood flow through adipose tissue (Jeukendrup et al., 1998a). During moderate exercise, arterial plasma fatty acid concentration can increase up to 20 times therefore increasing the resting ratio of FA/albumin from 0.2 to 3-4 during exercise at this intensity (Jeukendrup et al., 1998a).

Bülow (1982) concluded that the increase in subcutaneous adipose tissue blood flow during exercise is of importance for the enhanced FFA mobilization. Also of importance was the fact that subcutaneous adipose tissue blood flow response was found to be equal between both man and dog and that lipolysis, FFA mobilization and FFA reesterification are increased in subcutaneous adipose tissue during prolonged exercise (Bülow, 1982). The FA/albumin ratio is increased as well as the concentration of non-protein bound FA in plasma; a situation that favors reesterification (Bülow, 1982).

The extent of reesterification within the adipocyte includes the aforementioned ability of the plasma to carry away released FFA, but also with the availability of glucose to produce glycerol 3-phosphate for reesterification (Wolfe et al., 1990). As adipose tissue increases its utilization of glucose, the outflow of FFA decreases but glycerol release continues due to glycerol 3-phosphate, and a larger portion of glucose being converted to fatty acids (Murray et al., 2006). Conversely, as glucose utilization decreases, proportionally more glucose is shuttled to the formation of glycerol 3-phosphate for esterification of Acetyl-CoA which minimizes the efflux of FFA (Murray et al., 2006). In situations where there is not enough glucose available, less G-3-P will be formed and the rate of reesterification will decrease as a consequence along with greater amounts of fatty acids mobilized from the adipose tissue and released into circulation (Wolfe et al., 1990).

3) *Transportation of FA to the muscle cell by the blood.* Fatty acids either pass through the cell membrane of the adipocyte passively as most often the case with SCFA and MCFA or with the help of membrane associated proteins such as fatty acid

translocase (FAT) and fatty acid transport protein (FATP) (van Dam et al., 2004). Fatty acids then move through the interstitial fluid bound to albumin before passing through the vascular wall of the capillaries where they again bind to albumin (Jeukendrup et al., 1998a). Recall that a majority of all fatty acids are bound to albumin in the plasma and that lipoproteins other than VLDL which incorporate FA in the liver, can function as carriers for FA through the blood (chylomicrons, LDL, HDL) (Murray et al., 2006). Fatty acids must be released from albumin upon contact with the endothelial cells lining the wall of muscle capillaries as they have a low affinity for the albumin/FA complex; albumin is too large to pass through the interendothelial clefts (Bassingthwaite et al., 1989).

4) *Fatty acids are taken up by the muscle cell.* The uptake of fatty acids by the muscle cell was once thought to be a passive process, occurring solely by passive diffusion. There is now evidence that membrane associated carrier proteins are also involved in mediating this process (Wolfe, 1998; Campbell et al., 2004; Koonen et al., 2005). Uptake of fatty acids from the plasma would be initiated by their translocation through the luminal membrane of the endothelial cell, the cytoplasmic compartment of the endothelial cell and subsequently through the albuminal membrane of the endothelial cell (Jeukendrup et al., 1998a). Next, again probably bound to albumin, FA are transported through the interstitial space then transported through the sarcolemma either passively or with the aid of plasma membrane bound carrier proteins such as fatty acid binding protein (FABP_{pm}) (Kiens, 2006) or by fatty acid translocase (FAT) and fatty acid transport protein (FATP) (Wolfe, 1998; Kiens, 2006). Once in the cytoplasm, fatty acids

are carried from the sarcolemma and to the outer mitochondrial membrane bound to cytoplasmic FABP (FABP_c) (Kaikaus et al., 1990; Koonen et al., 2005).

5) *Mobilization of FA from intramuscular triacylglycerol pools: availability of plasma fatty acids, lipolysis in the muscle.* The use of fatty acids as a substrate is dependent upon lipolysis in adipose tissue, the transport of these fatty acids through the blood and ultimate uptake in the muscle; nonetheless, lipolysis in the muscle may also be a factor contributing to the use of fatty acids especially in endurance trained humans (Jeukendrup et al., 1998a). During the first few minutes of exercise, plasma fatty acid concentration decreases due to the delay between increased uptake and the stimulation of lipolysis (Wolfe et al., 1990). Once lipolysis is up and running, reesterification is suppressed and plasma fatty acid concentration will rise (Wolfe et al., 1990). Plasma substrate oxidation continues to progressively increase with increasing exercise duration (Romijn et al., 1993). Lipolysis of intramuscular TG does not appear to be stimulated at low exercise intensities, but it does appear to be stimulated at moderate intensities (65% $\text{VO}_{2\text{max}}$); however, it does not seem to increase with higher intensity exercise (85% $\text{VO}_{2\text{max}}$) (Romijn et al., 1993). At 65% $\text{VO}_{2\text{max}}$, Romijn et al. (1993) found that FA from lipolysis in adipose tissue and intramuscular stores contributed equally to fat oxidation. With high intensity exercise efforts (> 80% $\text{VO}_{2\text{max}}$), there is limited FA availability due to the decreased R_a FA release from adipose tissue (Romijn et al., 1993). Post-exercise, even though lipolysis decreases immediately, $[\text{FA}]_{\text{plasma}}$ continues to rise after exercise ceases indicating increased release from previously hydrolyzed TG (Wolfe et al., 1990;

Romijn et al., 1993). These high post-exercise plasma fatty acid levels are thought to replenish muscle TG as well as glycogen stores (Walker et al., 1991).

The rate of fatty acid oxidation in the working muscle is dependent upon several factors, not just the $[FA]_{\text{plasma}}$, but also the number and size of mitochondria, and the presence of other substrates such as glucose (Jeukendrup et al., 1998a). The hyperglycemia and insulinemia brought about by ingestion of a meal by horses prior to exercise will tend to decrease FA oxidation due to the insulin-induced reduction in lipolysis and the subsequent decrease in NEFA concentration in the plasma along with an initial, temporary decrease in plasma glucose at the onset of exercise (Lawrence et al., 1995; Pagan and Harris, 1999). For another, exercise activity at a high intensity will increase the rate of glycogenolysis and therefore glycolysis leading to increased muscle pyruvate concentration and ultimately lowering the rate of fat oxidation (Sahlin et al., 1990). High rates of fat oxidation require a minimum amount of TCA cycle intermediates (TCAI) and both at the onset of exercise and during the latter phases of prolonged exercise, TCAI levels seem to be maintained by the alanine aminotransferase reaction in the muscle (pyruvate + glutamate \leftrightarrow alanine + α -ketoglutarate) (Sahlin et al., 1990). However, an adequate rate of anaplerotic reactions cannot be maintained if muscle glycogen stores become depleted during prolonged exercise; as a consequence, glycogen depletion will lead to fatigue as muscle pyruvate concentration and therefore the number of TCAI fall below a critical level (Sahlin et al., 1990).

IMTG hydrolysis – hormone sensitive lipase. Although the regulation of TG synthesis and its subsequent breakdown is not completely understood, it is thought that hormone-sensitive lipase (HSL) is the major enzyme responsible for the hydrolysis of

stored TG in skeletal muscle, similar to adipose tissue (Kiens, 2006). Hormone-sensitive lipase is a multi-functional lipase that hydrolyzes triacylglycerol to form free fatty acids and glycerol; it is distinguishable from lipoprotein lipase which catalyzes lipoprotein triacylglycerol hydrolysis before its uptake into extrahepatic tissues (Murray et al., 2006). The liver and kidney take up the glycerol from the blood as both organs possess active glycerol kinase (Murray et al., 2006). In rat (Langfort et al., 1999) and human skeletal muscle, HSL protein has been detected, although in lesser quantities than in adipose tissue (Roepstorff et al., 2004b). Hormone-sensitive lipase is rate limiting in lipolysis of adipose tissue (Fredrikson et al., 1981) but not TG hydrolysis in skeletal muscle (Roepstorff et al., 2004b) suggesting that other lipases may be involved in TG hydrolysis. Further, HSL protein expression is higher in oxidative muscle fibers than in glycolytic fibers (Langfort et al., 1999).

Roepstorff et al. (2004b) cited a rapid, temporary increase in HSL activity at the onset of exercise with a decline in activity towards resting levels as exercise continued. This observation is contrary to the fact that exercise produces a continuous increase in catecholamine concentration, specifically epinephrine, which tends to increase HSL activity (Kiens, 2006). It is not completely understood how molecular mechanisms regulate HSL activity in skeletal muscle, but it appears that many factors could play a role in both adipose tissue and skeletal muscle HSL activity.

6) *Transport of FA into the mitochondria of the muscle cell.* When exercise intensity increases, thereby decreasing fatty acid oxidation, fatty acid oxidation is limited by factors inside the muscle cell (Campbell et. al, 2004). The rate of glycolysis which is

the regulator of carbohydrate metabolism in the muscle, contributes to the rapid mobilization of glycogen during high intensity exercise; thus, rapid carbohydrate oxidation (metabolism) inhibits fatty acid oxidation by limiting its transport into the mitochondria (Wolfe, 1998). Wolfe (1998) believes that the primary control of lipid metabolism in exercise is the latter of 1) release of FFA or 2) at muscle tissue level; because muscle is major site of control of the rate of fat oxidation during exercise, the rate of control is the entry of substrate into mitochondria.

Once fatty acids enter the cytoplasm of the muscle cell, they are either esterified and stored into the intramuscular TG (Dagenais et al., 1976) or bound to a transport protein such as FABP for delivery to the site of oxidation (Jeukendrup et al., 1998a; Kiens, 2006). Long chain fatty acids are activated in the cytosol in a reaction with CoA and ATP to yield a fatty acyl-CoA by long-chain acyl-CoA synthetase (ACS) (Jeukendrup et al., 1998a; Kiens, 2006). In addition to being located on the cytosolic surface of the peroxisomal endoplasmic reticulum and outer mitochondrial membranes (Coleman et al., 2000) long-chain acyl-CoA synthetase has been found to also be located on the plasma membrane (Gargiulo et al., 1999). The latter location seems to help maintain a low intracellular LCFA concentration because of efficient esterification at the plasma membrane (Kiens, 2006). Acyl CoA synthetase may therefore affect the rate and directionality of LCFA across the membranes along with other proteins that are involved in LCFA uptake (Gargiulo et al., 1999). It is generally accepted that short and medium chain fatty acids can more freely diffuse into the mitochondrial matrix where they are converted to their CoA esters (Jeukendrup et al., 1998a). The transport of LCFA moieties within the cytoplasm and subsequent donation to metabolic β -oxidation, is

mediated by the cytosolic Fatty Acid Binding Protein (FABP_c) family (Kaikaus et al., 1990) and acyl CoA binding protein (ACBP) (Rasmussen et al., 1994). This partnership is believed to be the major factor in controlling the free concentration of cytosolic LCFA-CoA even though they are relatively water soluble as very little LCFA and LCFA-CoA exist free or unbound (Kiens, 2006). Long chain fatty acids bound for oxidation must pass from circulation through the endothelium, the interstitial space, the plasma membrane, the cytosol and ultimately through the mitochondrial membranes where final oxidation takes place. What remains under debate is whether transendothelial or transsarcolemma transport of LCFA is a passive process dependent on cellular metabolism, or whether LCFA transport occurs via plasma membrane protein-mediated transport. It appears that both factors contribute to the transport of LCFAs (Bonen et al., 2000; Pownall and Hamilton, 2003).

The movement of LCFAs across the mitochondrial membranes was thought to, traditionally, be controlled only by the carnitine-palmitoyl transferase (CPT) system as the inner mitochondrial membrane is impermeable to fatty acids (or acyl-CoA). Carnitine palmitoyltransferase I, present on the outer mitochondrial membrane, catalyzes the trans-esterification of fatty acyl CoA to acylcarnitine to cross the inner mitochondrial membrane via the acyl carnitine/carnitine translocase system in a 1:1 exchange for free carnitine (Campbell et al., 2004; Kiens, 2006). On the matrix side of the membrane, acylcarnitine is reconverted to fatty acyl-CoA by CPTII (Campbell et al., 2004). Since important regulators of CPTI are found to only minimally affect its function, it has been suggested that fatty acid-binding proteins may be involved in the transport of LCFA into the mitochondria (Campbell et al., 2004). Recently, researchers have found three fatty

acid binding proteins on the plasma membrane (Campbell et al., 2004; Roepstorff et al., 2004a; Kiens, 2006):

1. plasma membrane-bound fatty acid binding protein (FABP_{pm})
2. fatty acid transport protein (FATP)
3. fatty acid translocase (FAT/CD36)

FABP_{pm}. Plasma membrane bound fatty acid binding protein (FABP_{pm}) is believed to play a role in the transport of LCFA across the sarcolemma. It is a 43-kDa protein located peripherally on the plasma membrane and it is identical to mitochondrial enzyme aspartate aminotransferase (mATT) (Roepstorff et al., 2004a). Dietary manipulations have been found to affect expression of the FABP_{pm} protein. For instance, while high-fat diets increased the protein expression of FABP_{pm} in the vastus lateralis muscle in male [human] volunteers, the opposite occurred with those subjects on a carbohydrate-rich diet – FABP_{pm} protein content decreased (Roepstorff et al., 2004a). Exercise training also increased protein expression of FABP_{pm}, but only in male subjects (Roepstorff et al., 2004a). Likewise, a significant positive correlation between percentage of muscle FABP content and percentage of oxidative fibers in rat skeletal muscle was found by Vork et al. (1991) indicating that FABP plays an important role in fatty acid oxidation.

FATP. Fatty acid transport protein (FATP) is expressed in adipose tissue, the heart, and skeletal muscle in rodents (Schaffer and Lodish, 1994) and skeletal muscle in humans (Kiens et al., 2004). It is a 63-kDa integral protein with 6 predicted transmembrane domains (Schaffer and Lodish, 1994). It has been shown to have acyl

CoA synthase activity role in conversion of fatty acids to fatty acid acyl CoA (Hall et al., 2003). High-fat diets caused an elevation in FATP1 protein content in rat soleus muscle, but a decrease in gastrocnemius muscle, while the opposite occurred with the high-carbohydrate diet (Marotta et al., 2004).

FAT/CD36. Fatty acid translocase or FAT/CD36 is an 88-kDa integral membrane glycoprotein used for uptake of LCFA which are found in the intracellular pools of skeletal muscle (Campbell et al., 2004; van Dam et al., 2004; Kiens, 2006) and translocates to the plasma membrane of skeletal muscle cells upon stimulation by insulin or contraction (Bonen et al., 1999; Bonen et al., 2000; Luiken et al., 2002) which correlates with increased palmitate uptake (Luiken et al., 2002). Researchers postulate that FAT/CD36 plays a role in LCFA transport in the mitochondria (Campbell et al., 2004; van Dam et al., 2004). Fatty acid binding protein (FABP) may cooperate with FAT/CD36 in the uptake of LCFA in cardiac and skeletal muscle (Luiken et al., 1999).

FAT/CD36 is more abundant in oxidative type I fibers than glycolytic type II fibers in skeletal muscle including rodent and equine skeletal muscle (Campbell et al., 2004; Van Dam et al., 2004). There exists two types of mitochondria in skeletal muscle defined as subsarcolemmal (SS) and intermyofibrillar (IMF) (Campbell et al., 2004).

Campbell et al. (2004) found that expression of FAT/CD36 was lower in white gastrocnemius (low oxidative muscle fibers) (30.9% lower in SS and 36.4% lower IMF) as compared to red gastrocnemius muscle (high oxidative fibers) from female Sprague-Dawley rats. In human vastus lateralis muscle, a fat-rich diet induced an increase in the expression of FAT/CD36 (Cameron-Smith et al., 2003; Roepstorff et al., 2004a). Moreover, IMTG content was found simultaneously with a high expression of

FAT/CD36 induced by the high-fat diets (Roepstorff et al., 2004a) and it could be ascertained that enhanced amount of FAT/CD36 would be of benefit for clearance of LCFA. Further support was given by van Dam et al. (2004) in which FAT/CD36 in equine skeletal muscle was found in close proximity to the sarcolemma, positioned near capillaries to aid in the uptake of LCFA from the plasma. Depending upon energy needs, LCFAs could then either be oxidized if energy was needed or used towards resynthesis into triacylglycerol (IMTG) if energy needs were low (Kiens, 2006). While diet seems to enhance FAT/CD36 expression (Roepstorff et al., 2004a), conditioning does not upregulate FAT/CD36 protein content in human subjects (Kiens et al., 2004).

Long-chain fatty acids are unable to be transported into the mitochondrial matrix, inhibiting further metabolism without the presence of carnitine. Likewise, palmitate oxidation was also dramatically reduced when a known inhibitor of FAT/CD36, sulfo-N-succinimidyl oleate (SSO) was incubated with viable mitochondria from rat muscle by 87 and 85% in SS and IMF (Campbell et al., 2004). As the need for greater fatty acid oxidation increases so does FAT/CD36 expression and thus capacity for fatty acid oxidation. As shown by Campbell et al. (2004) with both chronically and acutely electrically stimulated hind limb rat muscles.

Chronic stimulation, 7 days, of the hind limb rat muscles resulted in the up-regulation in total amount of FAT/CD36 present in the total homogenate and in the plasma membrane fraction of skeletal muscle along with an increase in the mitochondrial fraction as compared to control muscles. Therefore, mitochondrial palmitate oxidation was increased in the chronically stimulated muscles, but SSO reduced fatty acid oxidation in both experimental and control muscles (Campbell et al., 2004).

Acute stimulation for 30 min did not increase the total amount (expression) of FAT/CD36, but did increase amount in the plasma membrane fraction signifying that FAT/CD36 was translocated within the cell. Palmitate oxidation also increased while SSO again reduced fatty acid oxidation in both experimental and control muscles (Campbell et al., 2004). Investigators found no differences between SS and IMF fractions of skeletal muscle mitochondria. Palmitate binding and palmitate oxidation by mitochondria can be significantly altered by use of SSO, a known inhibitor of FAT/CD36. Further, FAT/CD36 quantity was in accordance with the oxidative capacity of the tissue: heart >> red muscle (high oxidative) > white muscle (low oxidative). Also, when rates of fatty acid oxidation increased, so did mitochondrial FAT/CD36 content due to either increased expression of FAT/CD36 as in chronic stimulation or by translocation of FAT/CD36 to the mitochondrial membrane as in the acute stimulation (Campbell et al., 2004). FAT/CD36 is likely helped by the carnitine-palmitoyl transferase system in the movement of LCFA across the mitochondrial membranes.

In a proposed model by Campbell et al. (2004) illustrating the interaction between FAT/CD36 and CPTI, FAT/CD36 acts as an LCFA acceptor from cytosolic-binding proteins and transferring them to the long chain acyl CoA synthetase which activates the LCFA, preparing it for CPTI acceptance. Perhaps this allows for another key regulatory step in fatty acid oxidation.

GLUT4. Although glucose and fatty acids are the primary metabolic fuel for the exercising muscle, their ability to be used is contingent upon their transport into the myocyte. The glucose transporter 4 (GLUT4) protein is almost solely responsible for the transport of glucose in mammalian muscle cells (Richter et al., 2001). In rats, GLUT4

protein has been found in intracellular subsarcolemmal groups of vesicles and between mitochondria and the myofibrillar region (Ploug et al., 1998) and is translocated from the storage sites by the stimulation of insulin and muscle contraction (Luiken et al., 2002; van Dam et al., 2004) as is the case with FAT/CD36. In equine skeletal muscle, a greater expression of the GLUT4 protein was observed in type 2B (IIB and IIX) fibers of various muscle types as compared to type 1 and 2A (IIA) (van Dam et al., 2004). In general, GLUT4 content in skeletal muscle decreased with increasing oxidative capacity of the muscle groups; however, some studies have cited a large amount of GLUT4 in type 1 fibers as well and that GLUT4 translocation does not differ between muscle types, but rather differs according to oxidative capacity (Marette et al., 1992). However, there seems to be no strict designation of GLUT4 expression and muscle fiber type among or between species; therefore, there seems to be alternative factors that contribute to GLUT4 expression in skeletal muscle such as insulin, muscle contraction and exercise training, along with diet (van Dam et al., 2004).

A high-carbohydrate diet increased both GLUT4 protein expression and mRNA in both type I and II muscle fibers in rats (Lee et al., 2002), but did not increase GLUT4 gene expression in horses after a glycogen depleting exercise (Jose-Cunilleras et al., 2005). Conversely, a high-fat diet was found to decrease GLUT4 expression in one study with rats (Kahn and Pedersen, 1993). On the other hand, while the GLUT4 protein expression was increased with exercise in another study while at the same time GLUT4 mRNA expression decreased with the high-fat diet, suggesting that both mRNA and protein expression is controlled independently by exercise and diet (Lee et al., 2002). It must be noted that the rats in the study by Kahn and Pedersen (1993) were obese. In

sum, it appears that skeletal muscle has the capacity to adapt to various stimuli with regards to fiber specific glucose uptake and expression.

Caveolins. Lipid rafts and caveolae, a morphological subclass of lipid rafts, have been suggested to play a role in fatty acid uptake. Caveolae and caveolin are involved in the regulation of lipid transport. Caveolae are 50 – 100 nm flask-shaped indentions of the plasma membrane composed of sphingolipids and cholesterol which contain caveolin, the protein which is essential for invagination of the plasma membrane (Kiens, 2006). Caveolin-1 and caveolin-2 are found in most cell types while caveolin-3 is muscle specific be it cardiac, smooth or skeletal muscle (van Deurs et al., 2003).

In conditions of fatty acid utilization, coordination of caveolin-1 and FAT/CD36 may be involved (Kiens, 2006). Researchers found an increase in expression of FAT/CD36 and caveolin-1 protein expression in human skeletal muscle during the recovery phase after intense, glycogen depleting exercise regardless of the amount of carbohydrate consumed (Roepstorff et al., 2004a). In addition, caveolae may regulate the function of fatty acid transporters such as FAT/CD36 (Kiens, 2006) researchers found a high degree of colonization of caveolin-3 and FAT/CD36 on the sarcolemma of human vastus lateralis muscle (Vistisen et al., 2004).

The effect of exercise on protein transporter activity. Exercise alone can increase LCFA uptake and oxidation many times over. In the rat, skeletal muscle uptake of palmitate into sarcolemmal giant vesicles increased 75% after 30 minutes of contractions correlating with the expression of FAT/CD36 (Bonen et al., 2000) as well as increased the number and/or activity of the lipid binding proteins in the trained versus untrained state, which may facilitate transport of LCFA into the myocyte (Turcotte et al., 1992;

Kiens et al., 1993). Kiens (2006) suggests that increased FAT/CD36 expression is an early adaptation to increased muscle activity that may fade with sustained increased activity, as short-term training (Tunstall et al., 2002; Pelsers et al., 2008) and even a single exercise bout (Roepstorff et al., 2004b) increased FAT/CD36 protein expression slightly. However, there was no difference in FAT/CD36 content in the vastus lateralis muscle when comparing endurance trained and untrained human subjects (Kiens et al., 2004). This also coincides with an increase in muscle FABP_{pm} and contraction-induced uptake and oxidation of palmitate in trained rats (Turcotte et al., 1999) but while training increased FABP concentration in the rat heart muscle, rat skeletal muscle concentration was not increased (van Breda et al., 1992). In contrast to diet, exercise did not induce changes in content of FAT/CD36, FABP_c or acyl-CoA binding protein (ACBP), suggesting that these transporters may occur in adequate amounts to transport LCFA and LCFA-CoA during exercise (Kiens et al., 2004).

Kiens (2006) cautions that even though there is evidence that transporters are involved in the transport of LCFA, and certain exercise conditions occur in which transmembrane transport during exercise may not be the important limiting step in plasma LCFA oxidation. This does not mean that the transport proteins or the transport process is rate limiting; rather, when exercise intensity increases and FA oxidation decreases, factors inside the muscle cell are responsible for limiting fat oxidation.

The effect of diet on protein transporter activity. Interventions resulting in changes in lipid metabolism, such as diet, can have an effect on content and regulation of the fatty acid binding proteins. In studies done in rats, data have shown that in heart and skeletal muscle, cytosolic fatty acid binding protein (FABP_c) did not respond to an

increase in dietary saturated fatty acids (Coe and Bernlohr, 1998; Storch and Thumser, 2000). However, a diet rich in omega-3 fatty acids significantly increased FABP_c content (Clavel et al., 2002), suggesting that both FA length and degree of saturation are important for FABP_c regulation (Pelsers et al., 2008). In a human study, consumption of a fat-rich diet was found to increase the expression of both plasma membrane fatty acid-binding protein (FABP_{pm}) as well as FAT/CD36 in the vastus lateralis muscle in contrast to a carbohydrate-rich diet (Roepstorff et al., 2004c). Likewise, gene expression of FAT/CD36 was greater after a high-fat diet as compared to an isocaloric equivalent high-carbohydrate diet in human skeletal muscle (Cameron-Smith et al., 2003).

7) *Oxidation of fatty acids occurs in the mitochondria.* Beta oxidation is the stepwise degradation of the fatty acyl-CoA which was transported into the mitochondria by the transport proteins with the help of the carnitine-palmitoyl transferase (CPT) system. The only step that requires energy from ATP is the activation of a fatty acid into an acyl-CoA by acyl-CoA synthetase (Murray et al., 2006). Each subsequent step in the oxidation process shortens the fatty acyl-CoA by two carbons; it is degraded to acetyl-CoA and an acyl-CoA residue. Acetyl-CoA can then enter the TCA cycle, thus following the same exact pathway as acetyl-CoA units from pyruvate, formed by glycolysis. The acyl-CoA unit will continue back through β -oxidation until it is completely oxidized. The rate of β -oxidation is dependent upon the type of fatty acid oxidized – both the number of carbons and the degree of saturation have been shown to influence oxidation rate (Jeukendrup et al., 1998a). In general, the longer the fatty acid chain length, the slower the rate of oxidation as very long chain fatty acids (C₂₀, C₂₂, etc.) must go through

a modified form of β -oxidation in the peroxisomes whose enzymes are induced by high-fat diets (Murray et al., 2006).

The enzymes of the citric acid cycle, β -oxidation, and the respiratory chain along with the mechanisms for oxidative phosphorylation are all contained within the mitochondria. Reducing equivalents produced from the citric acid cycle are collected and transported by the respiratory chain to their final reaction with oxygen to form water. The respiratory chain occurs in the mitochondria where food energy is converted to ATP; a proton gradient drives the synthesis of ATP (Murray et al., 2006). Oxidative phosphorylation is the process by which the liberated free energy is trapped as a high-energy phosphate (Murray et al., 2006).

Many conditions can control the rate of respiration in the mitochondria, namely the availability of ADP as oxidation and the associated phosphorylation of ADP are tightly coupled (Murray et al., 2006). The following lists states of respiratory control, adapted from Murray et al. (2006):

1. The availability of ADP and substrate
2. The availability of substrate only
3. The capacity of the respiratory chain itself when all substrates and components are present in saturating amounts
4. The availability of ADP only (rest)
5. The availability of O_2 only

At rest, the rate of respiration in most cells is dependent upon the availability of ADP only. However, when work is performed, ATP is converted to ADP which

increases respiration, but also replenishes ATP at the same time. As respiration increases, as in exercise conditions, the cell is then at state 3 or 5, when either the capacity of the respiratory chain becomes saturated or PO_2 decreases below K_m for heme a_3 . In addition, the ADP/ATP transporter, which facilitates entry of cytosolic ADP into and ATP out of the mitochondrion, could become rate-limiting (Murray et al., 2006). The remaining free energy that is not captured as HEP is liberated as heat which contributes to the maintenance of body temperature (Murray et al., 2006).

Effect of exercise, intensity and duration, on substrate utilization

The extent at which substrates such as carbohydrate, fat and protein are used by contracting muscles depends upon the intensity and duration of exercise, the availability of carbohydrate or alternative fuels, and the influence of hormones (Lawrence, 1990). Other factors that determine substrate usage include the activity of oxidative enzymes and the availability of O_2 for their complete oxidation and/ or density of mitochondria in the muscle, muscle composition, training status, as well as diet and exogenous substrates consumed before exercise (Pösö et al., 2004). In general, horses use carbohydrate fuel for high intensity, short duration exercise and fat for low intensity, long duration activities. Nonetheless, exercise intensity contributes the most to the determination of the degree of fat or carbohydrate oxidation during exercise (Jeukendrup et al., 1998b).

Generally speaking, at rest, when energy metabolism is fully aerobic, a majority of the energy requirements can be met by fatty acid oxidation as those reactions that require the least ADP can go the fastest; therefore, the oxidation of fatty acids will be preferred over oxidation of pyruvate because the former uses less ADP (McGilvery,

1975). However, as horse diets are generally low in lipid content, plasma NEFA concentration is usually low at rest. Therefore, horses will primarily use acetate and glucose as an energy source at rest (Pethick et al., 1993). With increasing exercise intensities, plasma glucose tissue uptake and muscle glycogen oxidation will increase, whereas the regulation of lipid metabolism is far more complex (Romijn et al., 1993a).

Fatty acid contribution to exercise comes from plasma FFA and muscle TG. At low intensity exercise (25% VO_{2max}) most of the energy requirements are met by fat, primarily plasma fatty acids in combination with glucose (Romijn et al., 1993a). As exercise intensity increases to 65% VO_{2max} , fat oxidation contributes to approximately 50% of energy expenditure - while plasma fatty acid contribution is lower, muscle substrate contribution such as IMTG and glycogen increase (Romijn et al., 1993a). Since the energy turnover is higher at this intensity, absolute fat oxidation rates are greater with the absolute rates of energy provision from fat being optimum between 50% and 70% VO_{2max} (Romijn et al., 1993a). In an experiment by Romijn et al. (1993a) five trained human subjects were studied during exercise intensities of 25, 65, and 85% of VO_{2max} . As indicated by the rate of appearance of peripheral glycerol, lipolysis was stimulated maximally at the lowest exercise intensity while rate of appearance of fatty acids in plasma decreased with increasing exercise intensity. Muscle triglyceride lipolysis was stimulated only at higher exercise intensities (65% and 85% VO_{2max}) as indicated by increasing intramuscular glycerol rate of appearance as the exercise intensity increased. The overall effect was a decrease in fat oxidation with increasing exercise intensity.

However, in contrast to plasma FFA, an important observation was that muscle triglyceride usage increased as exercise intensity increased; FFA oxidation at 85%

$\text{VO}_{2\text{max}}$ was not entirely dictated by a reduced capacity of the muscle to oxidize fatty acids. In addition, if peripheral and intramuscular fatty acid oxidation were summed, almost all of the available fatty acids were oxidized at the higher exercise intensities leading the researchers to question if the regulation site of fat oxidation at higher exercise intensities was in the adipose tissue itself (Romijn et al., 1993a).

Intramuscular factors as well could be responsible for decreased rates of fat oxidation during high intensity exercise such as the increased rates of glycolysis and acetyl-CoA formation from G-6-P could inhibit LCFA transport into the mitochondria at the level of CPTI by malonyl-CoA concentration (Sidossis et al., 1997). In addition, with increasing exercise intensity more fast twitch, type II glycolytic fibers are recruited which have less capacity to oxidize FA than slow twitch fibers (Snow et al., 1982; Essén-Gustavsson et al., 1984; Yamano et al., 2006). Likewise, there is increased competition into the citric acid cycle of pyruvate-derived acetyl-CoA as compared to fatty acid derived acetyl-CoA (Holloszy and Kohrt, 1996). As glycogen stores become depleted as with high-intensity exercise, carbohydrate oxidation falls below a critical level, but fatty acid contribution to exercise can compensate as long as fatty acids are available and the exercise intensity is reduced to a submaximal level, because the rate of ATP production decreases (Pernow and Saltin, 1971). Equine researchers have cited a significant decreased anaerobic power generation coinciding with glycogen depletion (Lacombe et al., 2001).

Also, it leads to the question that if more plasma FFA were available would it equal a greater fat oxidation? To test this theory, Romijn et al. (1995) lead another experiment illustrating that by acutely increasing the plasma FFA concentration in

conditioned humans at 85% VO_{2max} using six endurance conditioned male cyclists - once during control study and again when FFA concentration was maintained at 1 and 2 mM by intravenous infusion of lipid and heparin. The [FFA] failed to increase above resting levels in the control trial and plasma FFA concentration declined, consistent with results of their previous studies at this exercise intensity. By infusing lipid, FFA rate of appearances was almost ten-fold greater than the value during the control trial. In addition, this elevation resulted in a 27% increase in total fat oxidation. Still, the contribution of fat oxidation to energy expenditure increased to only 35% of the total at this exercise intensity. The rate of fat oxidation was still lower at 85% VO_{2max} than observed at 65% VO_{2max} in the previous study. Therefore, while low plasma FFA concentration may limit fat oxidation at times of strenuous exercise, other factors such as the efficiency of fat oxidation at the muscle level is the most likely controlling factor in regulating the rate of fat oxidation.

Conditioning

Research has shown that in both animals and man, adaptive increases in oxidative potential occur due to increased physical activity. Endurance training in human subjects increases the ability of the body to use fat as a substrate while at the same time, sparing muscle glycogen (Jeukendrup et al., 1997). Hurley et al. (1986) reports that the greater utilization of FFA found in their trained versus untrained human subjects was due to increased lipolysis of muscle TG. Nonetheless, Tyler et al. (1998) suggests that there may be an upper limit to the ability of training stimulus to evoke skeletal muscle adaptive responses in horses subjected to overtraining; adaptations beyond 16 weeks of training

were small and insignificant. Further, endurance or moderate intensity exercise appears to be more important for eliciting changes than high intensity exercise (Tyler et al., 1998). At the same time, McGowan et al. (2002) noted that, with high intensity training, of Standardbreds, a substantial increase in aerobic metabolic pathways occurred, but they did not see the same increase in anaerobic metabolic pathways due to conditioning. Factors that may contribute to stimulation of fat oxidation in trained subjects include an increase in number of oxidative enzymes and mitochondrial content in conditioned muscle, increased fatty acid uptake, an increase in muscle triacylglycerol content and oxidation, and alterations in mobilization of fatty acids from adipose tissue.

Conditioning effect on oxidative enzymes and mitochondrial content. As it is important for the trained muscle to have the increased ability to combust FA during exercise, conditioning could increase mitochondrial enzymatic activity. Endurance training increases the capacity to oxidize fatty acids as well as pyruvate and proportionally more FA and less carbohydrate are oxidized could be due to the conditioning effect of increased mitochondrial density in trained muscle (Gollnick and Saltin, 1982). The increased size and density of the mitochondria as an effect of conditioning, increases the surface area where the exchange of substrates and ADP can take place and possibly increase the number of transport proteins (Gollnick and Saltin, 1982). In Standardbreds, Tyler et al. (1998) observed an almost linear increase in mitochondrial volume throughout the conditioning period, greater than the changes in VO_{2max} , and cited a 131% increase in mitochondrial volume after conditioning. Further, the correlation between mitochondrial volume and VO_{2max} was stronger than that between mitochondrial volume and run time (Tyler et al., 1998). Gollick (1985) agrees that

increased endurance capacity and shift to fat metabolism is more pronounced than those changes in VO_{2max} in humans after endurance conditioning.

Enhanced activity of enzymes involved in fat oxidation is the prime cause of increased fat oxidation after training (Gollnick, 1985). Many studies have found increased levels of enzymes involved in the activation, transport, and oxidation of fatty acids. Beta-Hydroxy acyl CoA dehydrogenase (HAD) is a key enzyme in β -oxidation and its activity is shown to increase in times of FA flux other than exercise, like during ingestion of fat rich diets (Helge and Kiens, 1997). A positive correlation between LCFA uptake and HAD activity has been shown (Kiens, 1997). Beta-Hydroxy acyl CoA dehydrogenase activity increased 32% in horses after conditioning along with a 29% increase in citrate synthase (CS) activity corresponding with a decrease in muscle glycogen utilization (McGowan et al., 2002). Likewise in human subjects, HAD activity was greater in conditioned versus unconditioned subjects (Kiens et al., 1993). It could be speculated that enzyme activity is one of the main limitations in lipid oxidation in the exercising muscle cell, but the exact mechanisms are not clear.

Conditioning has also been demonstrated to increase the number of capillaries associated with muscle fibers as well as the percentage of oxidative fibers in humans (Kiens et al., 1993) and horses (Henckel, 1983; Serrano et al, 2000), theoretically allowing for a reduced diffusion distance and an increase in the surface area for exchange of substrates within the muscle (Kiens et al., 1993). While muscle oxidative enzyme activities rapidly decline after conditioning is ceased, changes in muscle fiber types, area and capillarization do not, presumably because the changes in muscle fiber characteristics are slow to occur (Tyler et al., 1998).

Other potential key regulatory steps in metabolism could be the the $[ATP]/[ADP]$ and the $[ATP]/[ADP] \times [P_i]$ ratios in the cytosol and mitochondria (Gollnick and Saltin, 1982). The higher mitochondrial concentration in the muscle, as occurs with conditioning, the more effective the translocation of ADP into the mitochondria; therefore decreasing the concentration of free ADP $[ADP_f]$ (Gollnick, 1985). The faster translocation of ADP as compared to ATP keeps the $[ATP]/[ADP] \times [P_i]$ ratio high in the cytosol, inhibiting phosphofructokinase (PFK). By maintaining a lower level of ADP and increasing the $[ATP]/[ADP]$ ratio, the increased ratio would favor greater entry of acetyl-CoA from fatty acids into oxidative pathways (Gollnick, 1985) while increased $[ADP_f]$ and a lower ratio would have the opposite effect, favoring glycolysis (McGilvery, 1975). When the supply of ADP becomes saturating, such as with high intensity efforts, those processes will be favored that consume the most ADP and the least oxygen (McGilvery, 1975).

In sum, conditioning increases mitochondrial content and density, decreases $[ADP_f]$ at certain work loads, increases the $[ATP]/[ADP]$ and $[ATP]/[ADP] \times [P_i]$ ratios, in which the overall effect is increased oxidative capacity.

Conditioning effect on plasma FA utilization. Many studies cite a lower plasma fatty acid concentration after training and this could be due to either a reduced mobilization from adipose tissue or an increased extraction of fatty acid by the muscle (Hurley et al., 1986). Even though fat oxidation is increased with training, it is unlikely that this increase is due to plasma fatty acids; training does not increase plasma fatty acid extraction by the muscle (Hurley et al., 1986). After conditioning, human volunteers reduced their reliance on plasma FA utilization while at the same time, increased their

use of intramuscular TG; indicated by a more than two-fold increase in the latter (Phillips et al., 1996). Thus, conditioning induced a decrease in adipose tissue lipolysis, while skeletal muscle lipolysis seems to increase (Hurley et al., 1986; Phillips et al., 1996). Reliance on blood glucose and muscle glycogen after conditioning are therefore reduced (Hurley et al., 1986). Conditioning lowered plasma LCFA concentration at rest and during exercise; however, with improved physical conditioning, plasma LCFA increased in horses – blood glucose followed similar pattern (Hambleton et al., 1980).

Conditioning effect on IMTG utilization. It was just mentioned that despite higher rates of fat oxidation during exercise, plasma fatty acid oxidation decreases, suggesting that additional fat sources used during exercise do not come from adipose stores, but possibly IMTG. Some studies cite an increase in IMTG concentration in trained human skeletal muscle (Hurley et al., 1986) making IMTG an important substrate for working muscle both in humans (Essén, 1977; Hurley et al., 1986; Kanaley et al., 1995) and rats (Fröberg, 1971; Spriet et al., 1986) signifying increased IMTG oxidation after training (Issekutz et al., 1964; Hurley et al., 1986; Phillips et al., 1996). In acutely exercised rats, red muscle TG concentration was significantly reduced after 3 hours of exercise (Fröberg, 1971). One study, involving a classical Swedish Wasa ski race [85 km], found that the most physically fit skiers had the largest IMTG stores before the race and also depleted them the most (Lithell et al., 1979). In the same study, while LPL activity increased only somewhat in the more fit skiers, there was a 6-fold increase in the less conditioned subjects, indicating a higher capacity for uptake of serum TG as compared to the more fit subjects (Lithell et al., 1979).

Conditioning can induce an increase in the number of lipid droplets located adjacent to the outer mitochondrial membrane as well. Tyler et al. (1998) noted a marked increase in intracellular lipid in horses after conditioning - lipid droplets increasing in number and size and lying in chains with mitochondria throughout type 1 fibers. Thus, transit time into the mitochondria would therefore theoretically be short. Likewise, Serrano et al. (2000) also documented an increase in intramuscular lipid deposits in horses coinciding with a decline in anaerobic enzyme activity with endurance conditioning.

It is important to note that some of the LCFA taken up in the exercising muscle is esterified to IMTG in non-active motor units especially at lower exercise intensities and could have influence on the data obtained especially when the indirect estimation of myocellular triacylglycerol utilization during exercise is used (Kiens, 2006). Kiens (2006) contends that sufficient data are not available to ascertain whether or not training increases IMTG utilization during exercise in humans and the differences could be explained by the wide range of dietary regimes in various studies. In conclusion, while training can potentially increase the IMTG pool as well as IMTG oxidation at the same absolute exercise intensity, the exact mechanism is not known.

Conditioning effect on plasma VLDL-TG utilization. Increased activity of LPL along with an increased capillary endothelial surface area due to conditioning, does not lead to increased hydrolysis of plasma triacylglycerols, rather VLDL-TG usage during exercise was found to be relatively small and inconsistent in trained versus untrained human subjects (Kiens et al., 1993). Lower carbohydrate utilization in the trained state is a function of reduced glycogenolysis [i.e. leads to sparing of muscle glycogen] of the

muscle; uptake of glucose by the muscle is unaffected by training, but muscle glycogen content is higher at rest in the trained subjects (Kiens et al., 1993). It was suggested that VLDL-TG degradation played a more important role in influencing blood lipid profiles than contributing to a higher rate of fat oxidation during exercise after training (Kiens et al., 1993).

Muscle LPL activity remains unchanged during moderate intensity exercise, however, activity increases during strenuous, prolonged exercise in humans, allowing for hydrolysis of VLDL-TG (Kiens, 2006). After glycogen-depleting exercise in humans, there remains an increased degradation of VLDL-TG and muscle TG. Post-exercise, resynthesis of glycogen is a priority, therefore FA liberated post-exercise due to increased LPL activity might be potential energy source during post-exercise recovery (Kiens and Richter, 1998). In horses however, glycogen repletion is very slow in comparison taking as long as 3 days or more (Hyypä et al., 1997) while human athletes can replenish much of their muscle glycogen stores in 24 hours (Goforth et al., 1997). Availability of substrates during recovery for energy provision as well as glycogen synthesis tends to affect the rate of repletion (Hyypä et al., 1997). Whereas plasma glucose levels were greater than or equal to pre-exercise values after repeated exercise bouts in horses, there exists low availability of lipid substrates for energy production 2 - 72 hours post-exercise which may contribute to the slow rate of glycogen repletion in horses (Hyypä et al., 1997).

In conclusion, physical conditioning results in adaptive changes that increase oxidative capacity in both animals and man, primarily at low to moderate intensities. An increase in fatty acid oxidation post-training is due in part to the increase in

capillarization, mitochondrial density, and an increase in oxidative enzyme activity. The supply of fatty acids into the mitochondria may be important as an increase in fatty acid oxidation after training could be primarily from IMTG sources and not adipose tissue derived fatty acids or circulating TG.

Effect of diet – nutritional interventions to exercise performance

Through dietary manipulation it is possible to elicit changes in fat oxidation. In many athletic species, changes in nutritional profiles have occurred, namely consumption of a high-fat diet or fasting, in order to manipulate fat oxidation which in theory would increase the availability of fatty acids thereby reducing glycogen breakdown, leading to enhanced performance. Under fasting conditions and short-term fat supplementation, muscle glycogen content was decreased overall and fatigue resistance was reduced in human athletes (Jeukendrup et al., 1998c). Chronic high fat diets may produce adaptive responses that prevent the aforementioned detriments to performance, but fat supplementation in human sports medicine shows concern due to increased weight gain and an increase in endogenous fat; therefore, primarily the focus is the potential “glycogen sparing effect” as a possible benefit to fat supplementation in the human athlete (Jeukendrup et al., 1998c). Likewise, for the equine athlete, fat supplementation serves to not only provide a safer means to increase the caloric density of the feed, but also to potentially enhance performance via a “glycogen sparing effect” along with other benefits such as decreased bowel ballast and decreased metabolic heat production. It was generally accepted that exercise capacity is directly related to muscle glycogen content in the muscle prior to exercise and that glycogen concentration is directly influenced by the

carbohydrate content of the diet (Bergström et al., 1967). However, short-term high-fat feeding may be an insufficient amount of time to induce adaptive responses to said diet whereas ingestion of a high-fat diet over a long period can decrease carbohydrate utilization and the induced carbohydrate shortage could be compensated by an increased contribution of fat to energy metabolism.

In human nutrition, ingestion of long-chain triacylglycerols during exercise is undesirable in that it may reduce gastric emptying and cause gastrointestinal upset. In addition, LCFA appearance in plasma is quite slow, entering systemic circulation in chylomicrons which contribute only minimally to exercise fuel. On the other hand, medium-chain triacylglycerols are rapidly oxidized when ingested, but the quantity must be limited as gastrointestinal distress can occur (Jeukendrup et al., 1998c). However, ingestion of LCT may replenish IMTG stores post exercise (Jeukendrup et al., 1998c). On the other hand, it has been suggested that MCT can be a valuable exogenous energy source when ingested during exercise in combination with carbohydrate (Jeukendrup et al., 1995), as MCT is more rapidly emptied from stomach and oxidized. Although the availability of MCT was high when ingested with carbohydrate (70%), the maximum amount of MCT able to be tolerated when ingested was approximately 30g and at that level, the amount of MCT contributing to overall energy expenditure was limited to between 3 and 7% (Jeukendrup et al., 1998c).

The ingestion or infusion of TG in the horse during exercise is not a viable strategy as the clearance of infused triglyceride in horses is very slow. The half-life of infused TG in the horse has been reported to be at least 10-fold greater than in other species (Moser et al., 1993).

Nutritional strategies for improving fat oxidation – fasting, time of feeding.

Fasting has been proposed to increase fat utilization, spare muscle glycogen and therefore improve exercise performance. In rats, short-term fasting was reported to increase plasma epinephrine and norepinephrine concentration, stimulate lipolysis and increases the concentration of circulating plasma FA therefore increasing fat oxidation and sparing muscle glycogen (Jeukendrup et al., 1998c). Koubi et al. (1991) also found decreased contribution of carbohydrates as an energy supply, hence sparing of muscle glycogen and increased β -oxidation in the fasted versus the fed rats; however, endurance capacity was similar between the two groups. In contrast, others have found increased run time to exhaustion coinciding with fasting in exercised rats (Dohm et al., 1983).

In human subjects, exercising at 70-75% VO_{2max} for 90min, a 24 hour fast caused much greater fat utilization in the first half of the exercise trial but as exercise persisted, fat became greater proportion of the energy expenditure in the fed subjects; ultimately, it was found that endurance capacity decreased at this exercise intensity (Dohm et al., 1986). Investigators also cited decreased insulin concentration while the concentration of circulating catecholamines increased, an increased concentration of plasma FA, and an increase in plasma glucose during first 30 min of exercise despite the 24 hour fast nearly depleting liver glycogen stores (Dohm et al., 1986). Serum FFA, glycerols, and the ketone body, β -hydroxybutyrate, were higher in the fasted state than the post-absorptive state both at rest and during exercise (Knapik et al., 1988). Muscle glycogen remained unaffected, but liver glycogen stores decreased as indicated by decreased R_a glucose during exercise after 3.5 days of fasting compared to post-absorptive state, without a decline in endurance capacity (Knapik et al., 1988). Dohm et al. (1986) speculated that

decreased pyruvate oxidation in muscle of fasted subjects could thus lead to glycogen sparing. Many factors can contribute to the disparity in the information such as physical condition of subjects, fasting duration, as well as exercise intensity and duration.

In the horse, fasting from 8 to 12 hours before exercise appears to result in utilization of fat as an energy source during exercise as indicated by the net disappearance of NEFA (Duren et al., 1999). Further, feeding horses 3 hours prior to exercise resulted in hyperinsulinaemia and therefore a drop in blood glucose - hence, interference with fat metabolism. However, fat-supplementation appeared to attenuate the latter results somewhat (Duren et al., 1999). Pagan and Harris (1999) also found that feeding grain with or without hay within 2 hours of exercise reduced FFA availability and increases blood glucose disappearance. Feeding forage alone within this time frame does not appear to interfere with FFA availability, but may reduce plasma volume and increase body weight due to gut fill (Pagan and Harris, 1999). The glycemic index of the meal fed after fasting can also alter substrate utilization during exercise. Jose-Cunilleras et al. (2002) found that by feeding corn after an 18 hour fast and before exercise as compared to feeding alfalfa hay or fasting alone, resulted in higher plasma glucose and serum insulin, lower glycerol and serum NEFA; thus, resulting in higher carbohydrate and lower lipid oxidation during exercise.

In sum, fasting increases the availability of lipid substrates thereby increasing fat oxidation at rest and during exercise. However, glycogen stores are not maintained during fasting which therefore impairs fatigue resistance and exercise performance.

Nutritional strategies for improving fat oxidation – The high-fat diet. As shift in substrate utilization can occur by changing the carbohydrate and fat content of nutrition.

In human nutrition, this ratio of substrates providing energy can be expressed as the food quotient (FQ) which is analogous to the respiratory quotient (RQ) (Knapik et al., 1988). Thus a high-carbohydrate diet would have a high FQ and consequently a high RQ in contrast to a high-fat diet having a low FQ as well as a low RQ. Findings using the muscle biopsy technique indicated that the decreased quantity of muscle glycogen in the high-fat versus the high-carbohydrate diets was the main factor in the lack of fatigue resistance during prolonged exercise in humans; in addition, glycogen repletion was slowed in those subjects consuming the high-fat diet (Bergström et al., 1967; Hultman and Bergström, 1967).

Rats adapted to a high fat diet for 4 weeks had no reduction in endurance as compared to their carbohydrate adapted counterparts (Conlee et al., 1990). The fat-adapted rats had similar run time to fatigue times than the rats on the carbohydrate diet both in the initial exercise challenge and in the second challenge that occurred after just 72 hours of recovery despite lower pre-exercise muscle and liver glycogen levels along with slower muscle glycogen repletion after the first exercise phase. However, when fat and carbohydrate adapted groups were switched to the opposite diet for 72 hours, the fat group had restored its liver glycogen levels fully (and nearly their muscle glycogen levels) then ran longer than the carbohydrate group while the carbohydrate adapted rats were unable to restore their glycogen levels while consuming the fat diet (Conlee et al., 1990). The authors suggest that part of the fat adaptation may involve increased storage and utilization of IMTG (Conlee et al., 1990).

As Conlee et al. (1990) pointed out, the fat-adaption in their study did not have an impact on performance of their rats, rather it was the short-term fat feeding in non-

adapted rats that provided a detriment to endurance capacity. Further, Simi et al. (1991) found considerable improvement not only in endurance capacity, but VO_{2max} as well in rats after 12 weeks of dietary fat adaptation combined with conditioning, as compared to those rats on a standard carbohydrate diet. The authors attribute the affect on performance to an increase in the number of oxidative enzymes along with decreased degradation of liver glycogen during exercise (Simi et al., 1991).

Short-term, high-fat consumption among human athletes has been related to impaired performance and lower resting muscle (and presumably liver) glycogen levels (Burke and Hawley, 2002). Muscle glycogen also typically decreases with long-term, high-fat diets in humans (Phinney et al., 1983; Lambert et al., 1994) but the concentration of IMTG increases, possibly due to an increased delivery of fatty acids into the muscles from TG-rich lipoproteins through elevated lipoprotein lipase activity perhaps in addition to blood-borne FFA (Kiens et al., 1987). On the other hand, 2-4 weeks of a high-fat, low carbohydrate diet markedly increased fat oxidation and reduced the utilization of muscle glycogen during submaximal exercise efforts (Phinney et al., 1983; Lambert et al., 1994), despite almost 50% lower muscle glycogen levels in the fat adapted group (Phinney et al., 1983). Likewise, Lambert et al. (1994) cited a 44% lower pre-exercise muscle glycogen concentrations after adaptation to a high-fat diet, but no differences between the fat and carbohydrate groups were found in sprint performance and time to exhaustion during high intensity exercise; however, time to exhaustion during moderate intensity exercise (approximately 60% VO_{2max}) was significantly longer on the high-fat diet. Not surprisingly, fat oxidation rates were also increased during exercise for the high-fat diet (Lambert et al., 1994).

Conversely, another study found that time to exhaustion during endurance training decreased on a high-fat diet and the detrimental effects of the fat diet lingered even after switching to a high-carbohydrate diet; leading the authors to conclude that a high-fat diet is detrimental to endurance training in human athletes (Helge et al., 1996). Furthermore, adaptations to a fat-rich diet in humans have been linked to increased plasma norepinephrine concentration and heart rate during submaximal exercise, this increase in sympathetic nervous system activity while training might be considered an indicator of higher cardiovascular demands in the fat-fed group and may have contributed to the reduction in endurance in that group (Helge et al., 1996). Other studies have also noted a perceived increase in effort of training when consuming fat-rich diets (Jansson and Kaijser, 1982; Burke et al., 2002; Burke and Hawley, 2002). What was initially viewed as “glycogen sparing” effects of performance after adaptations to a fat-rich diet, rather may be a downregulation of carbohydrate metabolism or even “glycogen impairment. Consequently, Burke and Kiens (2006) dismiss dietary fat adaptation strategies for use in human athlete endeavors due to the growing evidence that performance is actually impaired by dietary fat adaptation strategies and dismiss it as an ergogenic aid for conventional endurance and ultraendurance sports. Pelsers et al. (2008) echoes these comments by concluding that there is “still no convincing evidence that this dietary training regime provides exercise performance benefits, since most competitive athletes perform at such a high workload that endogenous carbohydrate remains the predominant fuel.” In addition, consumption of a high-fat diet tends to down-regulate glycogenolysis and thus may even impair high intensity performance capacity (Burke and Kiens, 2006).

In equine athletes as well as their human counterparts, muscle glycogen content has a direct impact on performance capabilities. The decreased availability of muscle glycogen accounts for diminished anaerobic power generation and capacity for high intensity exercise as muscle glycogen accounts for a large percentage of metabolic fuel, especially during high intensity exercise (Lacombe et al., 2001). While human athletes are capable of carbohydrate loading or maximizing their muscle glycogen stores prior to an athletic event, the same practice is not successful in horses as horses already have a very high capacity to store glycogen – 600 to 650 mmol/ kg dry muscle (Pösö et al., 2004). Muscle glycogen concentration reaching those levels in human athletes only occur after successful carbohydrate loading. The original protocol for carbohydrate loading in humans was detailed by Bergström et al. (1967) which consisted of a depletion of glycogen stores, followed by overcompensation. As such, consumption of a diet containing approximately 50% starch would be required to obtain the same results in horses as their human counterparts when it comes to glycogen loading (Geor, 2004). Feeding strategies for equine athletes have focused on increasing pre-exercise glycogen concentration or glucose availability, or alternatively optimizing glycogen resynthesis post-exercise (Geor, 2004).

In contrast to human athletes, at least 72 hours is required for restoration of muscle glycogen stores after strenuous exercise for those horses fed a diet rich in soluble carbohydrates (Lacombe et al., 2004). In addition, muscle glycogen synthesis can be hastened somewhat by administration of large doses of glucose (Lacombe et al., 2001) or by feeding a high glycemic index diet, compared with medium and low glycemic index diets, as blood glucose availability to skeletal muscle and insulin release are increased

(Lacombe et al., 2004). However, there are drawbacks to using such diets in horses to alter or promote glycogen storage as the equine species did not evolve on a diet high in soluble carbohydrates and thus experience digestive and metabolic limitations to high grain diets (Kronfeld et al., 1994). In addition, hydrolysable carbohydrate content of horse feeds is already quite high and to further increase the starch content would put them at risk for a variety of gastrointestinal dysfunctions including metabolic syndromes (Sprouse et al., 1987; Clarke et al., 1990). Therefore, while carbohydrate loading is effective in human athletes, attempts to perform the same results in horses lead to metabolic disorders such as exertional rhabdomyolysis (Kronfeld and Downey, 1981), founder, and colic (Sprouse et al., 1987; Lindberg et al., 2006).

Adaption to a fat-supplemented diet does not enhance glycogen repletion, and in fact, if horses are not adapted to a fat diet, and they are fed a fat-rich diet post exercise, muscle glycogen repletion was actually slowed (Hyypä et al., 1999), as there is less substrate (glucose) available for muscle glycogen synthesis. Horses did tend to have higher muscle glycogen levels at rest when on a fat-supplemented diet (3 weeks, 10% animal fat), but more of the glycogen was used during the exercise test in the fat-supplemented horses, suggesting that the glycogen was available for glycogenolysis and subsequent glycolysis during anaerobic work (Oldham et al., 1990). On the other hand, Hambleton et al. (1980) cited lower pre-exercise muscle glycogen concentrations with a diet of 16% fat and Pagan et al. (1987) cited lower liver and muscle glycogen levels at rest and after exercise with a diet of 15% soybean oil fed for one month. This would suggest that there might be an optimum level of fat-supplementation as well as an optimum time for diet adaptation, both of which are contentious issues.

Fat adaptation is defined by Kronfeld and Downey (1981) as the process of conditioning animals fed a high fat diet with the intention of enhancing fatty acid oxidation, hence sparing the utilization of muscle glycogen and blood glucose. In addition, fat adaptation encompasses a set of physiologic responses to the consumption of a high-fat diet during conditioning that present advantages to the horse during exercise (Harris and Kronfeld, 2003). Metabolic responses to exercise may require 10 to 12 weeks of fat adaptation (Griewe et al., 1989; Taylor et al., 1995). However, some digestive and metabolic changes in response to feeding supplemental fat have been found in as little as three to five weeks (Harkins et al., 1992; Orme et al., 1997; Pagan et al., 2002). Pagan et al. (2002) found that it took at least five weeks of fat adaptation for carbohydrate oxidation to decrease while fat oxidation increased. At the same time, Orme et al. (1997) found that plasma total lipase activity was elevated above baseline following just three weeks of fat adaptation while it took six to ten weeks for peak enzyme activity; as well, activity was no longer significant five weeks after withdraw of the fat supplemented diet. The previous statement was also validated by Dunnett et al. in 2002; the effects of the fat supplemented diet, namely the increase in fat utilization in this instance, also diminish within five weeks of withdrawing the diet. The precise amount of time it takes for a horse to become fully adapted to a fat-supplemented diet is unclear; however, the assumed benefits of the diet do appear to be dependent on its continued use.

Similar discrepancies occur pertaining to the optimum level of fat supplementation. The National Research Council (NRC) (2007) does not list a requirement for fat as a nutrient for horses with the exception of a suggested dietary minimum for linoleic acid of 0.5 percent of dry matter intake. However, the upper limit

of fat inclusion for soy oil is suggested to be 0.7 g/kg BW/d (NRC, 2007). Harris and Kronfeld (2003) suggest that horses can consume up to 4 cups or 800g vegetable oil per day (for a 500 kg horse) as long as they are adapted to such a diet over a two to three week period and all other nutrients remain balanced. While research diets can contain up to 20-25% of DE from fat (Dunnett et al., 2002; Pagan et al., 2002) that is considerably higher than what is commonly practiced by horse owners and trainers of about 4-15% of DE from fat (Geor, 2004). In addition, the level of fat added to commercial diets is often limited by poor pellet quality, greasy appearance (Geor, 2004; NRC, 2007) in addition to propensity for oxidation, resulting in rancidity and therefore spoilage (NRC, 2007). Geor (2004) goes so far as to recommend an upper limit to oil supplementation at 100g per 100kg body weight per day, which for a 500g horse would be 500 g of oil or about 2 ½ cups of oil per day (1 cup = ~ 200 g = ~ 1.67 Mcal) which would provide 18% of daily DE requirements for a horse in moderate work – assuming 23.3 Mcal/d required according to the NRC (2007). The NRC (2007) cautions that feeding fat, since it is so energy dense, could lead to excessive weight gain for those horses not able to utilize all of the energy that it provides, but the practice could be beneficial for those horses requiring a large amount of daily DE. The benefits of being able to reduce the amount of concentrate fed include the ability to increase fiber intake. A common substitution is 2 parts fiber and 1 part fat for 3 parts starch and sugar (Harris and Kronfeld, 2003).

Further, since fats and oils do not contain vitamins or minerals, adding fat to an existing diet without regard to balancing the total ration according to energy, protein, vitamin and mineral contents could lead to potential nutrient imbalances and consultation with a nutritionist is recommended (Harris and Kronfeld, 2003; Geor, 2004). It has also

been recommended that additional vitamin E be added to the ration to maintain antioxidant status. For example, adding 100 IU vitamin E per 100 mL of oil added unless the oil being used is rich in available antioxidants in addition to the basal level of 160 IU per kg DM intake (Harris and Kronfeld, 2003).

Hambleton et al. (1980) concluded that 11.6% dietary fat was the optimum level for maximum muscle glycogen content when fat was fed at levels of 4, 8, 12, and 16% over a 3 week aerobic training period *in a latin square design* and that muscle glycogen content fit a parabolic curve. Although mean values for muscle glycogen concentration were higher when the horses were on the 8% and 12% diets, statistically significant differences were not found. Also, an optimal range of 10 – 13% dietary fat intake as suggested by Kronfeld (1996) would yield 95% of peak muscle glycogen (280 mmol/kg dry tissue). Meyers et al. (1987) and Scott et al. (1992) cited that exercise capacity and muscle glycogen content could be altered if the diet contained 10% [animal] fat and fed for at least 3 weeks. Nonetheless, the increased concentration of muscle glycogen at rest resulted in significantly higher usage of muscle glycogen during exercise (Scott et al., 1992).

In summation, responses to a diet high in fat diet are species specific as some species have evolved consuming diets containing proportionally more fat. Glycogen remains the substrate of choice for athlete performance and the impact of a diet high in fat on glycogen usage and synthesis depends on the species, diet adaption, level of fat within the diet, and post-exercise meal consumption. The capacity for a species to utilize fat as a substrate for physical activity and athlete performance is also dependent upon adaptation for a certain length of time to a diet high in fat. The specific amount of time

required for this to take place, as well as the optimum level of dietary fat, depends upon not only the species but also the individual.

Ergogenic effects of feeding fat to horses

Over the past several years, supplemental fat has been added to equine diets in the hopes of enhancing athletic performance. However, that premise has been met with varying success. Comparisons between studies have been difficult due to the tremendous variation in research methods and often low statistical power due to so few animals being used in the studies. What seems to be undisputed, however, is that supplemental fat does increase the energy density of the diet, as fat (9.185 Mcal/kg on average for vegetable fat and tallow) contains more than twice the amount of DE as corn (3.88 Mcal/kg) or even molasses and sugarcane (4.06 Mcal/kg) (NRC, 2007). Management is then able to feed an overall decreased volume of feed, i.e., less starch, while maintaining or even increasing caloric intake (Lawrence, 1990; Harkins et al., 1992; Kronfeld, 1996; Lindberg et al., 2006). Less volume fed would ultimately mean less starch in the diet which could decrease the chance of metabolic disorders (Sprouse et al., 1987; Clarke et al., 1990; Lindberg et al., 2006). Kane et al. (1979) concluded that horses can digest up to 30% of the DE in the form of fat with no adverse effects such as digestive complications.

The reduction in DM intake and therefore bowel ballast could cause an improved power-to-weight ratio. The reduced weight in the large bowel is associated with less undigested fiber which retains about twice its weight in water (Kronfeld et al., 1990). But a large water reservoir could be beneficial to those horses performing endurance work due to an increased intestinal water and electrolyte reserve (Meyer, 1987). Faster

speed could be accounted for by lower body weight regardless of the mixtures of the energy sources required for a given power output: low-power = fat oxidation, medium power = glucose oxidation and higher power = glycolysis (Kronfeld et al., 1994). Fat oxidation power output is low due to the slow process of LCFA transport across membranes (Kronfeld, 1996). The fat-adapted animal's ability to sprint would require diminished glucose and glycogen utilization at the pyruvate and not the glucose-6-phosphate level (Kronfeld et al., 1994). A better power/weight ratio could contribute to the faster speeds recorded in Oldham et al. (1990) and Harkins et al. (1992) in which horses sprinted over distances of 600 and 1,600 meters. Harkins et al. (1992) reported that the fat-supplemented horses ran the 1,600-m an average of 2.5 s faster than those on the control ration. However, whether the effects were due to diet or conditioning is not clear as the study design allowed for 3 additional weeks of conditioning to take place prior to the fat-supplemented horses completing the exercise challenge. Furthermore, the fat-supplemented horses were not fed hay during their dietary treatment and the diet overall contained more starch (and fat) than the control diet (Harkins et al., 1992) lending support to the notion that improved times could be wholly or partly due to decreased bowel ballast. While Oldham et al. (1990) reported faster run times in the last two sets of the 600-m sprints for the fat-supplemented horses, the significance level used was $P < 0.09$, higher than what is commonly accepted as being statistically significant.

Eaton et al. (1995) did find a small, but significant increased run time to fatigue and maximal accumulated oxygen deficit (MAOD - calculated as the difference between accumulated oxygen demand and measured (accumulated) oxygen uptake) at an intensity equal to 120% VO_2max ; however, these changes also occur in response to conditioning in

and of itself (Hinchcliff et al., 2002). Likewise, no corresponding change in resting muscle glycogen content or glycogen utilization rate during exercise was noted (Eaton et al., 1995). Web et al. (1987) found that supplementing cutting horse diets with fat improved their performance and provided more energy for work when they were subjected to repeated bouts of 90 seconds of high intensity exercise. There again, the study design allowed for three additional weeks of anaerobic conditioning to take place before the fat-supplemented horses were tested.

If supplementing fat into the equine athlete's diet could enhance muscle glycogen stores and/ or spare existing stores, hardly anyone would disagree that this would constitute a potential enhancement of performance. However, as previously mentioned, there is no definitive answer as to whether or not fat adaptation has a positive affect on muscle glycogen content or utilization.

Fat-supplemented diets have been shown to increase muscle glycogen stores in Quarter Horses and Thoroughbreds (Meyers et al. 1987; Oldham et al. 1990; Scott et al., 1992; Julen et al., 1995). In addition, Harkins et al. (1992) found approximately a 16% increase in muscle glycogen levels in the middle gluteal muscle after only 3 weeks of fat supplementation, but those horses were not fed hay and overall starch content of the fat-supplemented diet was higher than the control. For another, muscle glycogen utilization during exercise was nearly twice the amount in the fat supplemented horses as compared to the controls during exercise (Oldham et al., 1990; Scott et al., 1992), but not all studies reported pre and post-exercise muscle glycogen concentrations. Meyers et al. (1987) reported no significant alteration of VO_{2max} between fat-supplemented horses (5 and 10%) and controls.

On the other hand, other researchers have found no change in muscle glycogen concentration in response to a fat-supplemented diet (Essén-Gustavsson et al., 1991; Eaton et al., 1995; Orme et al., 1997; MacLeay et al., 1999; Lindberg et al., 2006). Although not statistically significant, there was a trend for the high-carbohydrate diet to have higher muscle glycogen concentration than those on the high fat diet (MacLeay et al., 1999) and glycogen concentrations in horses affected with recurrent exertional rhabdomyolysis (RER) were 18% higher while on the high-carbohydrate diet compared to those on the fat-supplemented diet. Utilization of muscle glycogen was not affected by dietary treatment in the same study. At the same time, the high-carbohydrate diet in Essén-Gustavsson et al. (1991) showed significantly higher (12%) muscle glycogen concentrations as compared to glycogen concentrations for horses on an isocaloric fat diet.

Other research indicates a moderate decrease in muscle glycogen concentrations following a period of dietary fat supplementation (Pagan et al., 1987; Geelen et al., 2001a; Griewe et al., 1989). Geelen et al. (2001a) reported that the decrease in muscle glycogen was consistent and statistically significant across four different muscles - the heart, masseter, gluteus and semitendinosus. Soybean oil was added to the high-fat diets in the aforementioned study at the expense of an isoenergetic amount of starch plus glucose for the control diets.

An alternative strategy to increasing muscle glycogen could be to spare muscle glycogen by using dietary fat. Griewe et al. (1989) cited a glycogen-sparing effect with the fat-supplemented horses as indicated by a reduction in glycogen utilization during the exercise test. And since there were no corresponding increases in glucose or FFA

utilization, the alternate fuel was speculated to be muscle TG although it was not measured in this study (Griewe et al., 1989). On the other hand, Pagan et al. (1987) found no difference in glycogen utilization between fat-supplemented horses and controls. The difference between the two studies could be the time allowed for adaptation. Pagan et al. (1987) utilized a Latin Square design in which each horse was fed a different diet (altering carbohydrate, fat and protein amounts) for a period of one month each. In contrast, Griewe et al. (1989) fed the horses the same diet for 12 weeks.

There seems to be as many papers indicating an increase as there are exhibiting no change in muscle glycogen content or utilization; Orme et al. (1997) also conceded to the fact that there is a great variation among published research when it comes to resting muscle glycogen concentrations. Harkins et al. (1992) concludes that the “impact of fat added diet may reside more with influence of pre-exercise metabolite levels than with moderating either skeletal muscle glycogen stores or the rate at which this energy substrate is used.” Therefore, there is no conclusive evidence that fat supplementation is ergogenic with respect to increasing skeletal muscle glycogen content or utilization and higher concentrations of muscle glycogen no matter what the source, does not necessarily constitute greater utilization.

Another benefit to feeding a fat-supplemented diet to horses may be decreased metabolic heat production. Kronfeld (1996) found that a fat-supplemented diet not only reduced daily heat load by 5%, but also reduced feed intake by 22%, fecal output (bowel ballast) by 31%, and water requirement by 12%. Furthermore, Kronfeld et al. (1994) cited reduced dehydration in endurance horses on a fat supplemented diet. Scott et al. (1993) concluded that the substitution of fat for some of the soluble carbohydrate in

traditional concentrate rations lowered the heat of fermentation, hence lowering the thermal load. Kronfeld (1996) went on to explain that when fat is consumed over carbohydrate, less carbon dioxide is produced, lower venous carbonic acid results and the reduced metabolic acidosis that follows may reduce heat production in fat-adapted horses during an athletic event.

Fat-supplementation or conditioning? The question remains if positive performance attributes can be credited to dietary fat supplementation or are they strictly a result of natural adaptations to exercise? As was reviewed in this paper, conditioning itself brings about changes in both aerobic and anaerobic capacity. For instance, run time to fatigue and maximum oxygen consumption is increased (Eaton et al., 1999; Hinchcliff et al., 2002), along with activities of oxidative enzymes such as citrate synthase (CS) and hydroxy acyl dehydrogenase (HAD) (Henckel, 1983; Serrano et al., 2000; McGowan et al., 2002). For another, fatty acid oxidation increases with endurance conditioning due to increase in the number of mitochondria in trained muscles (Holloszy et al., 1977; Tyler et al., 1998) along with a decreased reliance on muscle glycogen and blood glucose utilization (Holloszy et al., 1977; Geor et al., 2002). Therefore, a decrease in reliance on carbohydrate utilization as a substrate is indicated by increased rate of disappearance (R_d) of glucose and decreased net rate of glycogen utilization as confirmed by a decreased RER at both moderate (Geor et al., 2002) and high intensity (Geor et al., 1999) exercise. Likewise, an increase in MAOD is also found with anaerobic conditioning (Hinchcliff et al., 2002). The aforementioned changes can be apparent in as little as two to six weeks post training depending upon the exercise intensity (Pösö et al., 2004). Similarly, conditioning itself is known to increase muscle glycogen stores (Hambleton et al., 1980;

Geor et al., 2002; Hinchcliff et al., 2002) and capillarization of all fiber types (Henckel, 1983; Tyler et al., 1998; Serrano et al., 2000).

Conclusion. In sum, short-term high-fat diets can increase availability of lipid substrates, but can also decrease glycogen storage, therefore possibly increasing fat oxidation at the expense of reduced fatigue resistance and a decline in exercise performance. Low carbohydrate diets may also lead to increased breakdown of muscle TG. The apparent benefits of dietary fat adaptation in horses may have more to do with exercise intensity than anything else. For instance, increased capacity for fat oxidation has been demonstrated at low to moderate intensity exercise (Dunnett et al., 2002; Pagan et al., 2002), but the effect of fat-supplementation on fuel selection at higher workloads is not readily apparent as the requirement for carbohydrate as a fuel source as speed is increased is both uniform and obligatory (Dunnett et al., 2002). The mechanism for the enhancement of high-intensity exercise in fat-adapted horses remains unclear, but may be due to increased activation of glycolysis and glycogenolysis (Pösö et al., 2004). Lawrence (1990) concludes that fat supplementation as it relates to performance is quite controversial and the effects on performance are unclear. For another, Pagan et al. (2002) also admits that “The degree to which fat adaptation affects substrate utilization has not been quantified.” This is after it was postulated that dietary fat supplementation increased glycogen stores and thus would lead to improved performance due to a greater amount of substrate availability (Harkins et al. 1992). Still yet, Geelen et al. (2001a) contends that feeding high-fat diets to horses enhances transport of fatty acids and oxidative capacity of aerobic muscles. However, metabolic adaptations to fat-supplementation remain unclear (Harkins et al., 1992). In addition, horses are individuals

and will have varying results with dietary adaptations as well as conditioning due to genetic factors, diet, conditioning, nutritional status and endocrine response during exercise. The contribution of fat and carbohydrate to exercise depends on interaction between exercise intensity and all of the above factors which may influence horse's individual metabolic response to exercise (Dunnett et al., 2002). Therefore, it is not presently possible to unequivocally conclude that dietary fat supplementation is ergogenic with respect to equine athletic performance.

Long-term effects of feeding fat, safety

While a majority of studies as it relates to horses and fat-supplementation are performed over a rather short duration, there seems to be no adverse effects of feeding fat to horses long-term. In order to evaluate long-term effects of feeding a fat-supplemented diet, Pagan et al. (1995) fed 2-year-old Thoroughbreds in training soybean oil equivalent to 12% of their DE. This study reported no adverse effects from the fat-supplemented diet and concluded that such diets can be safely fed for extended periods of time to horses in training. Saturated fat can be fed as safely as unsaturated fat for long periods as proved by Harris et al. (1999) in a study where 12% of the DE was supplemental fat in the form of unsaturated soy oil or saturated coconut oil was fed for 10 months to mature Thoroughbreds as an acclimation period, then 6 more months at 20% of the DE coming from the same sources. Zeyner et al. (2002) fed exercising mature Warmblood-type horses high-starch or high-fat (16.3% soybean oil) diets for 390 days with no disadvantages noted in the fat diet over the starch diet.

CHAPTER III

METHODOLOGY

Animals and Diets

Seventeen Quarter Horse yearlings (6 geldings and 11 fillies) with no previous conditioning were utilized in a randomized complete block design to evaluate the effects of diet and exercise on substrate utilization and transporter expression in the skeletal muscle of horses. Animal procedures were approved by Oklahoma State University's Animal Care and Use Committee.

Acclimation period. Yearlings were housed on native grass pastures and were fed a basal diet consisting of corn, wheat, alfalfa meal and soybean meal for at least 30 days in order to acclimate them to the farm environment and allow for compensatory gain prior to the onset of the experimental periods. All horses were familiarized with a mechanical walker with a working diameter of 13 m (Pro Walk Manufacturing Company, Ada Oklahoma, model number 263-21).

Horses had a mean age of 15 mo with a range of 13 to 17 mo and a mean initial body weight of 359 kg upon commencement of the research trial. Horses were randomly

assigned to one of two treatment groups (Fat or Starch) and were divided into two, 8 week experimental periods containing 8 and 9 horses, respectively.

Health. All horses were given complete physical exams by licensed veterinarians prior to the start of the study to verify their health and ability to be involved in a conditioning program. Any horse with conditions that would place it at risk for injury or illness during the study was replaced with a more suitable individual prior to trial commencement. All horses were dewormed, given routine hoof and teeth care, and provided routine immunizations during the acclimatization period. The health of the horses was monitored at all times by visual assessment, visits from licensed veterinarians, as well as by daily rectal temperature readings taken at the morning feeding. Resting heart rate was also assessed by thoracic auscultation or digital palpation of the facial artery.

Housing. Horses were housed in 3.7 X 3.7 m box stalls bedded with pine shavings at the Oklahoma State Animal Sciences Equine Center. All horses were fed at 07:30 and 16:30 daily and any uneaten feedstuffs were weighed and recorded immediately prior to the morning feeding (feed was not removed except immediately prior to this feeding). All horses had free access to fresh clean water. Feeders and water buckets were cleaned out once daily and disinfected at least once per week.

Exercise conditioning. All horses were placed on an exercise program performing walk/trot sets for 30-40 minutes per day, four days per week for a period of 8 weeks.

Horses walked at 50% walker speed = 2 m/sec (4.5 mph); trotted at 90% walker speed = 3.2 m/sec (7.2 mph) to a target heart rate of about 110-120 bpm, monitored by using a wireless heart rate monitor (Kruuse Televet®, United Kingdom). Horses were turned out in dry lots two days per week for 2-3 hours of free exercise on those days when no conditioning was taking place; the remaining day was spent on stall rest.

Exercise challenge. At the conclusion of the 8 week aerobic conditioning program, all horses underwent an aerobic exercise test individually consisting of 20 minutes of uninterrupted trotting on the mechanical walker at a maximum speed of 4 m/sec (9 mph) while wearing the heart rate monitor. The target heart rate was 150 bpm during the exercise challenge.

Diets. Treatment diets (Table 2) consisted of two different pelleted (1.27 cm diameter) grain mixtures (Fat or Starch) that were fed at levels to equalize caloric intake. Rations were formulated to meet NRC recommendations for moderate growth and light exercise for yearling horses (NRC, 2007). The high-fat ration consisted of grain and roughage in a 60:40 ratio with the roughage portion consisting of Prairie hay and alfalfa cubes in a ratio of 30:10. Horses on the high-fat diet were fed at a level of 2.25% of their body weight. The high-starch ration consisted of grain and roughage in a 70:30 ratio with the roughage portion consisting of Prairie hay and alfalfa cubes in a ratio of 20:10. Horses on the high-starch diet were fed at a level of 2.50% of their body weight.

Table 2. Composition and chemical analysis of treatment diets

Item ¹	Fat (%)	Starch (%)
Corn, ground	15.50	33.00
Wheat middlings	21.00	30.00
Soybean meal	12.00	4.65
Limestone	1.00	2.00
Trace mineralized salt	0.25	0.35
Dicalcium phosphate	0.25	---
Vegetable oil	10.00	---
Alfalfa cubes	10.00	10.00
Prairie hay	30.00	20.00
Analysis ²	Fat	Starch
DM, %	91.05	89.16
DE, Mcal/kg	3.05	2.56
CP, %	13.79	12.32
ADF, %	19.58	16.39
NDF, %	30.53	27.03
Starch, %	22.75	39.22
Fat, %	12.99	2.84
Ca, %	0.89	1.20
P, %	0.32	0.30
Mg, %	0.16	0.16
K, %	0.94	0.83

¹As-fed basis

²DM basis

Sample Collection

Fecal collections. The eight week trial consisted of two, 72-hour collection periods on days 9-11 (Initial) and days 53-56 (Final) during which complete fecal collections were performed. The total fecal collection was weighed and multiple subsamples were taken every 24 hours during each collection period and frozen for later analysis.

All bedding was removed and horses were positioned on rubber mats in the stalls during each 72-hour collection period; horses remained tied during this time and hand-walked for exercise. Feces were collected from each horse and placed in individual collection tubs. At each 24-hour interval, collected feces were mixed and samples were taken to make a composite sample that represented a consistent percentage of each day's output; a total of three samples were collected per period. Samples were placed in pre-labeled Ziplock® bags and frozen immediately until analysis was performed.

Blood collection. Blood samples were taken three times throughout the experimental period by venipuncture of the jugular vein for analysis of glucose, lactate, triglycerides, ketone bodies (acetoacetate and betahydroxybutyrate), and hormones (insulin, leptin, adiponectin). The baseline blood samples (Time 1) were taken pre-feeding prior to the commencement of the research trial, day 1, and again after the treatment period, day 46 (Time 2). The third and final blood collection was taken immediately post-exercise, day 47 (Time 3). Approximately 25 ml of blood was taken

per horse per sampling time in pre-labeled tubes. Samples were allowed to clot at room temperature, centrifuged, and the serum or plasma frozen (-80°C) for later analysis.

Muscle biopsies. Muscle biopsies were performed to measure monocarboxylate transporters 1 and 4 (MCT 1 and MCT 4), glucose transporter 4 (GLUT4), and fatty acid translocase/CD36 (FAT/CD36) protein expression. Horses were placed in stocks and muscle biopsy samples were taken three times throughout the experimental period from the middle gluteal by licensed veterinarians. The baseline biopsy samples were taken pre-feeding prior to the commencement of the research trial, day 1 (Time 1) and after the treatment period, day 46 (Time 2) from horses that had rested overnight. The third and final biopsy, taken the day of the exercise challenge, was taken immediately post-exercise, day 47 (Time 3). Horses were fasted overnight prior to biopsy except for the post-exercise biopsy.

For the biopsy procedure, horses were placed in stocks where a small sample of gluteal muscle was obtained in the standing, awake horse by licensed veterinarians. Mild sedation of horses was undertaken with a single dose (0.25 – 0.30 ml) of dormosedan (10mg/ml). A small patch of hair (2x2cm) was clipped using size 40 surgical clipper blade from the gluteal region one-third of the way along an imaginary line drawn from the tuba coxa to the tail head. The site was scrubbed with Nolvasan and alcohol and 6-10ml of local anesthetic (2% Lidocaine) was infused into the skin and subcutaneous tissue. The sterilized site was covered with an iodine infused drape (Ioban) and a stab incision (1cm in length) was made using a size 15 scalpel blade. A 6 mm diameter U.C.H. (University College Hospital) Skeletal Muscle Biopsy Needle (Zask International

Medical supply, Alexandria, VA) was inserted through the incision to a depth of approximately 8cm from the skin surface into the middle gluteal muscle for a mix of oxidative and nonoxidative muscle fibers. A small sample of muscle was removed in the cutting window of the needle and pressure was applied to the wound. The small incision was sealed with tissue glue. A maximum of 3 biopsies per site were performed with an average of approximately 20-40 mg of wet muscle with each biopsy. Samples were frozen immediately in liquid nitrogen.

Parameters of growth. Weekly skeletal measurements were taken during the 8 week trial including wither, shoulder, knee, hip and hock using a standardized measuring tape. In addition, weekly body weights were taken using a calibrated livestock scale.

Body weight – the weight was recorded in a single weighing prior to the morning feeding and recorded to the nearest pound for later conversion to kilograms.

Wither height – the vertical distance from the ground to the highest protruding thoracic vertebrae in centimeters (cm)

Shoulder height – the vertical distance from the ground on the anterior side of the hoof to the point of the shoulder in cm

Knee height – the vertical distance from the ground on the anterior side of the hoof to the end of the distal radius in cm

Hip height – the vertical distance from the ground to the highest point of the croup in cm

Hock height – the vertical distance from the ground to the point of the hock (calcaneus)

Sample Analysis

Feed analysis. Hay, grain, and alfalfa pellets were sampled to measure nutrient content. Grab samples from each feed batch were mixed and the composite sample was sent in duplicate to Dairy One Forage Testing Laboratory (Ithaca, New York) for analysis of crude protein, fiber, fat, starch, calcium, phosphorus, magnesium and potassium. In addition, bomb calorimetry performed by Hazen Research, Inc. (Golden, Colorado) was used to determine gross energy values of feed samples from which digestive energy was calculated.

Fecal analysis. Fecal samples previously frozen were thawed overnight at room temperature. Composites were then mixed together per horse per collection period, and placed in drying ovens at 60°C for 72 h. Samples were then weighed and ground through a No. 3 Wiley Mill using a 2 mm screen. Initial and final sample weights were used to convert wet weights to a dry matter basis. Samples were then sent to Dairy One Forage Testing Laboratory for analysis of crude protein, fiber, fat, starch, calcium, phosphorus, magnesium and potassium, and to Hazen Research, Inc. for bomb calorimetry analysis in which gross energy values of fecal samples were used to calculate digestible energy.

Blood analysis. Blood analysis was performed using the GM7 Micro-Stat multiassay analyzer from Analox Instruments (Lunenburg, Massachusetts) for glucose,

lactate, triglycerides, acetoacetate and betahydroxybutyrate. Radioimmunoassay kits were used to analyze for the hormones Insulin, Leptin and Adiponectin.

Insulin. Concentrations of insulin in equine plasma were determined by using a commercial solid-phase radioimmunoassay (RIA) kit (Insulin Coat-a-Count; Diagnostics Products Corporation [Siemens] Dallas, Texas) previously validated for use in horses (Freestone et al., 1991) with a sensitivity of 1.2 μ IU/mL.

Samples, performed in duplicate, were allowed to come to room temperature and subsequently vortexed prior to pipetting 200 μ l into pre-labeled, coated tubes for the assay procedure. In addition, 4 plain (uncoated) polypropylene tubes T (total counts) and NSB (nonspecific binding) were labeled in duplicate. Fourteen insulin Ab-Coated Tubes A (maximum binding) and B through G were labeled in duplicate. One mL of 125 I insulin was added to every tube then vortexed, and allowed to incubate for 24 h at room temperature. Tubes were decanted following incubation and counted for 1 min in a gamma counter (Packard Instruments [PerkinElmer], MA).

Leptin. Determination of concentrations of leptin in equine plasma was performed by using a multi-species leptin radioimmunoassay kit (Millipore [Linco] Chicago, Illinois) as previously validated for use in horses (McManus and Fitzgerald, 2000; Cartmill et al., 2003; Kearns et al., 2006) with a sensitivity of 1.0 ng/mL. The kit employed 125 I-labeled recombinant human leptin with a specific activity of 135 μ Ci/ μ g, a guinea pig multi-species leptin primary antibody, and a goat anti-guinea pig IgG serum for the precipitating reagent. Kit standards and quality controls utilized purified

recombinant human leptin in assay buffer. Results are expressed as human equivalents of immunoreactive leptin (ir-leptin HE) due to the absence of purified equine leptin.

For the procedure using borosilicate glass tubes, 12 X 75 mm, assay buffer was pipetted into the non-specific binding (NSB) (300 μ l), reference tubes (200 μ l), and all remaining tubes, including samples (100 μ l). Standards, quality controls, and samples were performed in duplicate with sample tubes requiring 100 μ l of serum each. The multi-species leptin antibody was then used in all tubes except total count and NSB tubes. All tubes were vortexed, covered, and allowed to incubate for 24 h at 4°C.

On day two of the experiment, 100 μ l of 125 I-Human Leptin was added to all tubes followed again by a 24 hour incubation at 4°C. On day three, 1.0 mL of cold (4°C) Precipitating Reagent was added to all tubes except Total Count tubes, incubated for 20 minutes at 4°C, then centrifuged at 3,000 rpm for 20 minutes at 4°C. Tubes were then decanted and counted on a gamma counter (Packard Instruments [PerkinElmer], MA) for one minute.

Adiponectin. Plasma adiponectin concentrations were measured using a commercial radioimmunoassay kit (Millipore [Linco] Chicago, Illinois) previously validated for use in horses (Gordon and McKeever, 2005; Kearns et al., 2006). The adiponectin RIA assay utilizes 125 I-labeled murine adiponectin with a specific activity of 67.7 μ Ci/ μ g, a multispecies adiponectin rabbit antiserum, and a goat anti-rabbit IgG serum in the precipitating reagent to determine the level of Adiponectin in plasma by the double antibody/PEG technique. In the absence of purified equine adiponectin, results are expressed as human equivalents of immunoreactive adiponectin (ir-adiponectin HE). Sensitivity of the assay was 1.0 ng/mL.

All samples were diluted 1:500 with assay buffer prior to use. For the procedure using borosilicate glass tubes, 12 X 75 mm, assay buffer was pipetted into the non-specific binding (NSB) (300 µl), reference tubes (200 µl), and all remaining tubes, including samples (100 µl). Standards, quality controls, and samples were performed in duplicate with sample tubes requiring 100 µl of diluted (1:500) plasma each. Subsequently 100 µl of ¹²⁵I-Human Adiponectin tracer was added to all tubes plus 100 µl adiponectin antibody was then used in all tubes except total count and NSB tubes. All tubes were vortexed, covered, and allowed to incubate for 24 hours at room temperature.

On day two of the experiment, 10 µl of rabbit carrier was added along with 1.0 mL of cold (4°C) precipitating reagent to all tubes except total count tubes. Tubes were then vortexed and incubated for 20 minutes at 4°C before centrifuging at 3,000 rpm for 20 minutes at 4°C. Tubes were then decanted and counted on a gamma counter (Packard Instruments [PerkinElmer], MA) for four minutes.

Western blot analysis. Western Blot analysis was performed to evaluate monocarboxylate transporter (MCT) 1 and 4, glucose transporter 4 (GLUT4) and fatty acid translocase/CD36 (FAT/CD36). Approximately 50 mg of frozen muscle was homogenized briefly in 50mM Tris buffer containing 0.25 M sucrose, 0.01 mg/ml trypsin inhibitor, and 10 µg/ml phenylmethylsulphonyl fluoride (PMSF), pH 7.5 using a Con-Torque Tissue Homogenizer (Eberbach Corporation, MI). The cell homogenate was then cleared by centrifugation at 24,476 g for 15 min at 4°C and the supernatant containing cytosolic proteins from the muscle was recovered for protein determination

and Western immunoblotting analysis. Protein concentration of samples was determined by the Bradford Method (Wiley, 1999).

The samples were analyzed for transporter (FAT/CD36, GLUT4, MCT1, MCT4) protein content by Western blot analyses as described previously (Maiti et al., 2004). Equal amounts of protein were mixed with 4X sample buffer (Invitrogen, CA) containing 7-13% by weight of lauryl alcohol sulfate, lithium salt, plus dithiothreitol (DTT) and were then separated by SDS-PAGE using a Bio-Rad Mini-PROTEAN Tetra system (Bio-Rad, CA) on a 12% (w/v) polyacrylamide gel with running buffer for one hour at 155 V. Separated protein bands were electrophoretically transferred using the same system, to Polyvinylidene Fluoride (PVDF) membranes (Millipore, MA) overnight at 35 V.

The proteins were detected by blocking the membrane in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% (v/v) Tween 20) containing 5% (w/v) non-fat dried milk powder for one hour on a shaker at room temperature followed by an overnight incubation at 4°C with a polyclonal primary antibody. After incubation, membranes were washed with TBST for 4 x 15 minutes and incubated for two hours at room temperature with a horseradish peroxidase conjugated secondary antibody.

For FAT/CD36 protein detection, a goat polyclonal primary antibody was used (diluted 1:100) (Santa Cruz Biotechnology, Inc., CA) which was raised against a peptide mapping near the N-terminus of CD36 fatty acid transporter of human origin followed by two hour incubation with a horseradish peroxidase conjugated anti-goat immunoglobulin G secondary antibody (diluted 1:1000) (Santa Cruz Biotechnology, Inc., CA).

For the GLUT4 transporter, a goat polyclonal primary antibody was also used (diluted 1:100) (Santa Cruz Biotechnology, Inc., CA) which was raised against a peptide

mapping near the C-terminus of GLUT4 glucose transporter of human origin followed by incubation with a horseradish peroxidase-labeled anti-goat immunoglobulin G secondary antibody (diluted 1:2000) (Santa Cruz Biotechnology, Inc., CA).

For MCT1 detection, a rabbit polyclonal primary antibody (diluted 1:500) (Chemicon® International, (Millipore, MA) which was raised against a 15 amino acid sequence near the C-terminus of human MCT1 was used, followed by incubation with a horseradish peroxidase-labeled anti-rabbit immunoglobulin G secondary antibody (diluted 1:2000) (Thermo Fisher Scientific, PA).

For MCT4 transporter detection, a goat polyclonal primary antibody was used (diluted 1:500) (Santa Cruz Biotechnology, Inc., CA) which was raised against a peptide mapping near the C-terminus of MCT4 lactate transporter of human origin followed by incubation with a horseradish peroxidase-labeled anti-goat immunoglobulin G secondary antibody (diluted 1:2000) (Santa Cruz Biotechnology, Inc., CA).

Protein bands were visualized using a chemiluminescent substrate (Thermo Fisher Scientific, PA) according to manufacturer's instructions and by scanning by the Versa Doc™ Imaging System (Bio-Rad, CA). The intensity of the bands was quantified using the Quality One ® 1-D Analysis Software (Bio-Rad Laboratories, CA).

After the measurement of CD36 and MCT4, the membranes were stripped with buffer (2-mercaptoethanol, 10% SDS, 0.5M Tris·HCL pH 6.8) for 30 minutes at 55°C for subsequent analysis of GLUT4 and MCT1.

Statistical Analysis

Data collected during the first week of the study were used as baseline measures and diets were compared using analysis of covariance (ANCOVA) methods when the baseline measure is significantly related to the response variable modeling of the correlation (covariance) structure of the measurements across time as appropriate for the growth variables, heart rate, blood metabolites, hormones and transporters. For the digestibility variables, no covariate was necessary, and a repeated measures analysis modeling correlation structure was again done. The SAS/MIXED (2003 SAS Inst. Inc., Cary, NC) procedure was used for these analyses. All tests were performed at the $\alpha = 0.05$ level of significance.

CHAPTER IV

RESULTS AND DISCUSSION

Digestibility

Dry matter (DM). Data for DM intake, fecal output and DM digestibility are shown in Table 3. There was no treatment effect on DM intake ($P=0.362$), fecal output ($P=0.931$), or DM digestibility ($P=0.209$). There were period effects on fecal output ($P=0.001$) and DM digestibility ($P=0.008$), meaning that there was a consistent change from period I to period II across both treatments; fecal output increased, whereas digestibility decreased for both treatments.

The lack of an associative effect of fat supplementation on DM digestibility found in the current study agrees with several previous studies in horses (Webb et al., 1987; Meyers et al., 1987; Swinney et al., 1995; Bush et al., 2001; Kronfeld et al., 2004). Average digestibilities for Fat (68.16%), and the Starch treatment (70.01%), in the current study are higher than the 48.2% reported by Meyers et al. (1987) and the 54.7% reported by Bush et al. (2001). The present results are closer to the values obtained by Rich et al. (1981) who reported DM digestibility at 73.0% for a diet containing 10% corn oil. While a few studies cited differences in DM digestibility with diets containing added

fat (Kane et al., 1979; Jansen et al., 2000, 2002), a majority of the evidence supports no difference in DM digestibility between fat supplemented horses and controls.

See the following page for Table 3.

Table 3. Effects of a high-fat diet (Fat) and high-starch diet (Starch) on daily DM intake, fecal output, and DM digestibility (least squares means \pm SEM)

Response	Treatment ^a	
	Fat (n = 8)	Starch (n = 9)
Period I		
DMI, g/d	7115.92 \pm 285.06	7358.90 \pm 268.75
Fecal output, g/d	2196.80 \pm 127.79	2049.87 \pm 120.48
DM digestibility, %	68.94 \pm 1.75	72.42 \pm 1.69
Period II		
DMI, g/d	7259.93 \pm 247.02	7667.76 \pm 232.89
Fecal output, g/d	2361.34 \pm 100.73	2483.20 \pm 94.97
DM digestibility, %	67.38 \pm 1.65	67.60 \pm 1.60

^aMeans within a row did not differ ($P > 0.05$).

Crude fat. Data for crude fat intake, output and digestibility are shown in Table 4. Crude fat intake, output, and digestibility were greater ($P<0.01$) for the Fat treatment during both periods.

The higher digestibility of fat found in the Fat treatment agrees with other published equine studies (McCann et al., 1987; Scott et al., 1989; Julen et al., 1995; Bush et al., 2001; Lindberg et al., 2006). Bush et al. (2001) along with Lindberg and Palmgren Karlsson (2001) reported that the relationship between fat intake and fat digestibility is linear. For instance, Bush et al., (2001) reported fat digestibilities of 47.9% for control horses, 64.3% for horses supplemented with 5% corn oil and 75.9% for those supplemented with 10% corn oil. Likewise, Kane et al. (1979) reported mean digestibilities of 76.63, 89.02, and 90.90% for diets with 0, 15, and 30% added corn oil respectively. Their observation of the linear relationship between fat intake and digestibility is supported by the results of others (Rich et al., 1981; Meyers et al., 1989; Scott et al., 1989).

The mean digestibility percentage for the fat added diet in the current study (86.39%) is higher than some published reports (Meyers et al., 1987 - 73%; Bush et al., 2001 – 75.9%), but in line with others (Web et al., 1987 - 82.6%; Rich et al., 1981 – 81.4%) for the same fat inclusion level. The higher digestibilities observed in the Fat treatment versus the Starch treatment could be partially explained by the dilution of endogenous fecal fat by the supplemental fat, thus, enhancing apparent digestibility values (McCann et al., 1987). Furthermore, the magnitude of difference in digestibilities between the two treatment diets might suggest that the supplemented fat in the Fat treatment was more readily digested than the grain fat in the Starch treatment (Kane et

al., 1979; McCann et al., 1987). The availability of fat within cereal grains or oilseeds for digestion in the small intestine may be limited by encapsulation; in addition, the poor digestibility of waxes, pigments, and other non-triglyceride lipid components may contribute to the low true digestibility of forage fat (NRC, 2007).

See the following page for Table 4.

Table 4. Effects of a high-fat diet (Fat) and high-starch diet (Starch) on daily crude fat intake, output, and digestibility (least squares means \pm SEM)

Response	Treatment	
	Fat (n = 8)	Starch (n = 9)
Period I		
Crude fat intake, g/d	924.62 ^a \pm 23.52	204.12 ^b \pm 22.17
Crude fat output, g/d	131.19 ^a \pm 8.25	77.48 ^b \pm 7.78
Crude fat digestibility, %	85.79 ^a \pm 3.66	61.91 ^b \pm 3.56
Period II		
Crude fat intake, g/d	943.14 ^a \pm 20.83	212.89 ^b \pm 19.64
Crude fat output, g/d	123.61 ^a \pm 8.05	92.44 ^b \pm 7.59
Crude fat digestibility, %	86.98 ^a \pm 2.75	55.90 ^b \pm 2.73

^{a,b}Means within a row without a common superscript differ ($P < 0.05$).

Starch. Data for starch intake, output and digestibility are shown in Table 5. The Starch treatment had greater starch intake ($P<0.001$) along with a trend for higher starch output ($P=0.055$). There was no difference in starch digestibility between the two treatments.

As was the case with fat content in the treatment diets, the Starch treatment was formulated to have higher starch content; therefore, net starch intake was greater for horses in the Starch treatment group.

The digestion of starch over the total digestive tract of horses is almost complete and did not differ between different sources of starch according to Potter et al. (1992a). The mean apparent digestibilities of starch for both treatments in the current study were identical at 99.31% and confirm previous equine research (Hintz et al., 1971; Householder, 1978; Potter et al., 1992a).

See the following page for Table 5.

Table 5. Effects of a high-fat diet (Fat) and high-starch diet (Starch) on daily starch intake, output, and digestibility (least squares means \pm SEM)

Response	Treatment	
	Fat (n = 8)	Starch (n = 9)
Period I		
Starch intake, g/d	1619.25 ^a \pm 177.18	2991.66 ^b \pm 173.81
Starch output, g/d	12.00 \pm 4.57	17.61 \pm 4.31
Starch digestibility, %	99.25 \pm 0.178	99.40 \pm 0.167
Period II		
Starch intake, g/d	1653.09 ^a \pm 169.82	3004.16 ^b \pm 167.16
Starch output, g/d	10.23 \pm 5.33	22.92 \pm 5.03
Starch digestibility, %	99.37 \pm 0.214	99.21 \pm 0.202

^{a,b}Means within a row without a common superscript differ ($P < 0.05$).

Crude protein (CP). Data for CP intake, output and digestibility are shown in Table 6. There was no difference between treatments for CP intake ($P=0.263$); however, the Starch treatment had greater CP output ($P=0.003$) but lower digestibility ($P<0.001$) as compared to the Fat treatment.

The higher digestibility of CP observed in the Fat treatment is consistent with some published research in horses (Webb et al., 1987; Scott et al., 1989; Davison et al., 1991; Julen et al., 1995; Lindberg et al., 2006) but inconsistent with other research that found no difference with respect to CP digestibility between fat supplemented horses and controls (Rich et al., 1981; McCann et al., 1987; Meyers et al., 1987; Swinney 1995; Bush et al., 2001; Kronfeld et al., 2004).

Davison et al. (1991) suggests that the higher apparent CP digestibilities in their fat added diet is likely the result of the diluting effects of metabolic fecal nitrogen due to higher absolute protein intakes. Julen et al. (1995) echoed the aforementioned statement, although did not indicate differences in CP intake. Scott et al. (1989) theorizes that the increase in protein digestion with the 10% added fat diet in their trial is likely not reflective of an increase in true CP digestibility, but rather may be from the dilution of metabolic fecal nitrogen as the protein intake increased from 13.4% in their control diet to 17.5% in the diet with 10% added fat. It is also possible that the protein in the diet with 10% added fat was more digestible even though CP intakes were similar (Scott et al., 1989). Lindberg et al. (2006) suggests that the improved digestibility in their high-fat oats could have been due to their higher CP content. The percentage of CP of the Fat diet in the current study was only slightly higher at 13.79% versus 12.32% in the Starch diet (see Table 2), but there was ultimately no difference in CP intake between the two

treatments ($P=0.263$). However, protein quality is a function of amino acid profile and foregut digestibility (NRC, 2007) and a greater portion of the CP fraction of the Fat ration was composed of soybean meal which is highly digestible (Potter et al., 1992b; Farley et al., 1995). Furthermore, the mean CP digestibility for the fat diet is 74.72% which is consistent with digestibilities of 70-78% reported for soybean meal (Potter et al., 1992b; Farley et al., 1995). Likewise, the mean CP digestibilities in the current study (61.18% for controls and 74.72% for fat-supplemented horses) are consistent with Scott et al. (1989) who reported digestibilities of 63.7 % for control horses and 75.2 % for those supplemented with 10 % animal fat. The higher CP digestibility for horses on the Fat treatment found in the current study is likely due in part to a higher quality protein source even though total tract % digestibility is consistent with previous research.

See the following page for Table 6.

Table 6. Effects of a high-fat diet (Fat) and high-starch diet (Starch) on daily CP intake, output, and digestibility (least squares means \pm SEM)

Response	Treatment	
	Fat (n = 8)	Starch (n = 9)
Period I		
CP intake, g/d	981.76 \pm 47.38	946.80 \pm 45.65
CP output, g/d	244.50 ^a \pm 30.82	345.12 ^b \pm 29.06
CP digestibility, %	74.97 ^a \pm 2.92	63.68 ^b \pm 2.75
Period II		
CP intake, g/d	1001.58 \pm 45.40	928.06 \pm 43.83
CP output, g/d	256.17 ^a \pm 21.86	381.35 ^b \pm 20.61
CP digestibility, %	74.46 ^a \pm 1.90	58.67 ^b \pm 1.79

^{a,b}Means within a row without a common superscript differ ($P < 0.05$).

Energy. Gross energy intake, output, energy digestibility, and digestible energy are shown in Table 7. There were differences between treatments for energy intake ($P=0.024$) as well as digestible energy ($P=0.026$); the Fat treatment had higher mean responses for both. The interactions between period and treatment group were significant for gross energy output ($P=0.046$) and energy digestibility ($P=0.044$). For gross energy output, the Starch treatment had a lower mean response for Period I only. For digestibility, the Period II mean for the Starch treatment was significantly lower than all other means.

Diets were fed to equalize energy intake between treatments. However, the fat diet ultimately contained a greater amount of DE and thus absolute energy intake was greater for the horses on the Fat treatment. The higher energy content of fat as compared to starch has been documented. According to the NRC (2007) vegetable oil contains 9.19 Mcal/kg DE and tallow has 9.18 Mcal/kg DE whereas ground corn contains just 3.88 Mcal/kg DE. Kane et al. (1979) reported an increase in the density of DE with the addition of two levels of corn oil without a significant difference in energy digestibility when compared to the control ration. Further, Rich and coworkers (1981) found that energy digestibility was not affected by the type or the level of fat.

The lack of a difference in the digestion of energy found in period I between control and fat supplemented rations also agrees with several other studies (Webb et al., 1987; Meyers et al., 1987; Davison et al., 1991; Hughes et al., 1995; Swinney et al., 1995; Bush et al., 2001). Webb et al. (1987) reported no difference in energy digestibility in race horses or cutting horses supplemented with 10% fat. Other studies did report differences in energy digestibility between fat supplemented horses and controls as in

period II of the current study (Scott et al., 1989; Rich et al., 1981; Jansen et al., 2000). Scott et al. (1989) reported a significant difference in energy digestibilities for horses consuming a diet with 10% added animal fat diet (65.6%) and those on a control ration (61.4%). On the other hand, Jansen and coworkers (2000) reported that energy digestibility in horses decreased with adding fat to the diet.

Differences between mean energy digestibilities in the current study were not significantly different between the two diets although means were slightly higher for those horses fed the Fat treatment. Similar results were found by Meyers et al. (1987) in which mean energy digestibility for horses on the 10% fat-added diet was 56.4% while control horses had a mean energy digestibility of 54.3%. There are many discrepancies reported in equine research with regard to the effect of fat supplementation on energy digestibility. The different results reported by various studies concerning an associative effect of fat supplementation on energy digestibility could be due to many factors such as study design, length of time for fat adaptation, and individual variation.

See the following page for Table 7.

Table 7. Effects of a high-fat diet (Fat) and high-starch diet (Starch) on daily energy intake, output, digestible energy, and energy digestibility (least squares means \pm SEM)

Response	Treatment	
	Fat (n = 8)	Starch (n = 9)
Period I		
Gross energy intake, Mcal/d	32.26 ^a \pm 1.20	28.43 ^b \pm 1.13
Gross energy output, Mcal/d	10.56 ^a \pm 0.595	8.94 ^b \pm 0.561
Digestible energy, Mcal/d	21.70 ^a \pm 1.01	19.51 ^b \pm 0.962
Energy digestibility, %	67.09 \pm 1.67	68.79 \pm 1.59
Period II		
Gross energy intake, Mcal/d	32.92 ^a \pm 1.07	29.55 ^b \pm 1.01
Gross energy output, Mcal/d	10.75 \pm 0.498	10.68 \pm 0.470
Digestible energy, Mcal/d	22.17 ^a \pm 0.958	18.88 ^b \pm 0.915
Energy digestibility, %	67.30 ^a \pm 1.48	63.77 ^b \pm 1.41

^{a,b}Means within a row without a common superscript differ ($P < 0.05$).

Fiber. Intake, output, and digestibility values for acid detergent fiber (ADF) and neutral detergent fiber (NDF) are shown in Table 8. The Fat treatment had higher mean responses for both ADF intake ($P=0.009$) and ADF output ($P=0.006$) across both periods. There were no differences in ADF digestibility or any of the parameters (intake, output, and digestibility) for NDF between the two treatments. Period effects were evident for ADF intake, ADF digestibility, NDF output, and NDF digestibility ($P < 0.004$) meaning that the change from period I to period II for both treatments was consistent. ADF intake and NDF output response means were lower in Period I than in Period II, and digestibility for both ADF and NDF decreased from Period I to II. The interaction between period and treatment group was significant for ADF output ($P=0.041$) and NDF intake ($P=0.033$) response variables. The Fat treatment had greater ADF output during period I, while there was no difference in ADF output between the two treatments for period II. Furthermore, horses on the Fat treatment had greater NDF intake during period I; however, there was no difference between diets for NDF intake for period II.

The analysis of the treatment diets revealed that the fat ration contained a slightly greater percentage of ADF, 19.58% compared to 16.39% in the starch ration (see Table 2). Furthermore, horses on the fat treatment were receiving 10% more roughage in the form of prairie hay which contains a higher percentage of ADF and NDF fractions when compared to alfalfa cubes.

Mean digestibilities for ADF and NDF revealed a lack of an associative effect of fat supplementation on fiber digestion which is in agreement with other studies in which there was no difference in ADF digestibilities (Kane et al., 1979; Rich et al., 1981 [Exp

1]; Kronfeld et al., 2004) NDF digestibilities (Meyers et al., 1987; Swinney et al., 1995; Davison et al., 1991) or both ADF and NDF digestibilities (McCann et al., 1987; Bush et al., 2001). On the other hand, other researchers such as Rich et al. (1981) found an increase in the apparent digestibility of both ADF and NDF for peanut oil diets which was presumably due to the higher digestibility of the peanut oil itself. Webb et al. (1987) also reported an increased NDF digestibility in a diet with 10% added fat. Swinney et al. (1995) further defined an upward trend in NDF digestibilities in diets up to 15% fat, but found that the trend for NDF digestibility actually decreased in diets comprised of more than 15% fat. Jansen and coworkers (2000, 2002) reported that supplemental fat depresses fiber digestibility and while the exact mechanism is not understood, it could be due in part to poor adaption to the oil-supplemented diets. The lack of consistency in published research with regard to the effects of fat supplementation on fiber digestibility could be due to many factors which were not part of the investigations of the present study.

See the following page for Table 8.

Table 8. Effects of a high-fat diet (Fat) and high-starch diet (Starch) on daily fiber intake, output, and digestibility (least squares means \pm SEM)

Response	Treatment	
	Fat (n = 8)	Starch (n = 9)
Period I		
ADF intake, g/d	1393.34 ^a \pm 49.66	1170.39 ^b \pm 46.82
ADF output, g/d	759.10 ^a \pm 60.17	567.21 ^b \pm 59.52
ADF digestibility, %	45.02 \pm 3.90	51.47 \pm 3.84
Period II		
ADF intake, g/d	1421.37 ^a \pm 44.79	1273.18 ^b \pm 42.23
ADF output, g/d	850.58 \pm 68.46	803.39 \pm 67.01
ADF digestibility, %	39.73 \pm 4.57	36.73 \pm 4.45
Period I		
NDF intake, g/d	2173.07 ^a \pm 78.97	1924.17 ^b \pm 74.46
NDF output, g/d	1131.70 \pm 102.95	983.51 \pm 100.41
NDF digestibility, %	47.43 \pm 4.51	49.28 \pm 4.41
Period II		
NDF intake, g/d	2216.92 \pm 72.01	2114.67 \pm 67.89
NDF output, g/d	1286.28 \pm 99.84	1272.33 \pm 97.57
NDF digestibility, %	41.76 \pm 4.28	39.87 \pm 4.19

^{a,b}Means within a row without a common superscript differ ($P < 0.05$).

Calcium and phosphorus. Intake, excretion, and digestibility values for calcium (Ca) and phosphorus (P) are shown in Table 9. There were differences between treatments for Ca excretion ($P < 0.001$), and Ca digestibility ($P = 0.013$); the Starch treatment had higher mean excretion values, and therefore lower Ca digestibility. There were no treatment effects for P in any of the parameters, intake, excretion, or digestibility. There were period effects for Ca excretion ($P = 0.024$), P intake ($P = 0.003$), and P excretion ($P = 0.014$) meaning that Period II means were larger for all three of these variables for both treatments. The interaction between period and treatment group was significant for Ca intake ($P = 0.008$); the Starch treatment had higher mean responses in both periods, and from Period I to II there was an increase in calcium intake for the Starch treatment that was not evident in the Fat treatment.

An increase in the digestibility of Ca in the fat supplemented diet is in agreement with McCann et al. (1987) who reported that a diet composed of 15% corn oil had an increase in Ca absorption. The biological significance of the higher Ca digestibility of corn oil as compared to other fats or no fat in their study is not understood as serum Ca levels were within normal range. On the other hand, Rich et al. (1981) reported that the apparent absorption of Ca was not affected by the addition of fat to the diet nor was it affected by the type or level of fat added to the diet. Serum levels of Ca were also not affected by fat supplementation in the same study (Rich et al., 1981). Serum mineral analysis was not performed in current study and the reason for the higher Ca digestibilities seen in the Fat treatment is undetermined; however, it may have something to do with Ca source and/or fiber content of the diet.

Limestone content was the same (1.20%) for all three fat treatments in the study by McCann et al. (1987). In the current study, limestone composed 2.00% of the Starch diet and 1.00% of the Fat diet; the remainder of the Ca in the fat diet consisted of 0.25% dicalcium phosphate (see Table 2). The Ca in dicalcium phosphate is more highly available (73% versus 67%) than the Ca in limestone (Schryver, 1975) and even though Ca intake in the Starch treated horses was higher, excretion was also higher – efficiency of absorption increases at lower levels of Ca intake and decreases at higher levels (Schryver, 1975). In addition, lysine facilitates Ca absorption (Schryver, 1975) and the Fat diet contained a higher percentage (12% versus 4.65%) of soybean meal (see Table 2). Furthermore, horses on the Fat treatment in the current study were consuming 10% more fiber than those on the Starch treatment (see Table 2). Pagan et al. (1998) reported a higher Ca digestibility in a forage diet (59.3%) versus a mixed forage plus concentrate diet (48.3%). Whether Ca source or diet composition impacted Ca digestibility in the current study is not known.

The digestibilities of 54.78% for the Fat treatment and 46.11% for the Starch treatment are well within the range for the absorption efficiency of 50% stated by the NRC (2007) even though Ca digestibility was statistically different between the two dietary treatments.

The lack of an associative effect of fat supplementation on P digestibility confirms previous research in the equine species (McCann et al., 1987; Rich et al., 1981; Davison et al., 1991). There was no difference in P absorption between treatments in the study by McCann et al. (1987). At the same time, Rich and others (1981) reported that

the apparent absorption of P was not affected by the addition of fat to the diet nor was it affected by the type or level of fat added to the diet. As was the case with Ca, serum levels of P were also not affected by fat supplementation (Rich et al., 1981). True P absorption in horses is 30-55% according to the NRC (2007) which is similar to the P digestibilities reported in the current study of 33.76% for the Fat treatment and 31.04% for the Starch treatment.

See the following page for Table 9.

Table 9. Effects of a high-fat diet (Fat) and high-starch diet (Starch) on daily calcium and phosphorus intake, excretion, and digestibility (least squares means \pm SEM)

Response	Treatment	
	Fat (n = 8)	Starch (n = 9)
Period I		
Calcium intake, g/d	63.03 ^a \pm 3.07	86.28 ^b \pm 2.90
Calcium excretion, g/d	26.86 ^a \pm 3.28	45.95 ^b \pm 3.10
Calcium digestibility, %	57.40 ^a \pm 3.71	47.02 ^b \pm 3.54
Period II		
Calcium intake, g/d	64.29 ^a \pm 2.70	95.31 ^b \pm 2.54
Calcium excretion, g/d	30.57 ^a \pm 1.80	52.16 ^b \pm 1.70
Calcium digestibility, %	52.16 ^a \pm 2.84	45.20 ^b \pm 2.74
Period I		
Phosphorus intake, g/d	22.83 \pm 0.858	21.35 \pm 0.809
Phosphorus excretion, g/d	14.58 \pm 0.872	14.00 \pm 0.822
Phosphorus digestibility, %	35.94 \pm 3.62	34.49 \pm 3.43
Period II		
Phosphorus intake, g/d	23.29 \pm 0.767	23.20 \pm 0.723
Phosphorus excretion, g/d	15.92 \pm 0.678	16.75 \pm 0.640
Phosphorus digestibility, %	31.57 \pm 2.70	27.59 \pm 2.57

^{a,b}Means within a row without a common superscript differ ($P < 0.05$).

Magnesium and potassium. Intake, excretion, and digestibility values for magnesium (Mg) and potassium (K) are shown in Table 10. There were no treatment effects on the intake, excretion, or digestibility of Mg or K. There were period effects for Mg intake ($P=0.007$), Mg excretion ($P=0.002$), and Mg digestibility ($P=0.021$), as well as K intake ($P=0.022$), meaning that there was a consistent change from period I to period II for both treatments; Mg intake, Mg excretion and K intake increased from period I to period II across treatments while Mg digestibility decreased. The interaction between period and treatment group was significant for K excretion ($P=0.012$) and K digestibility ($P=0.023$), meaning that the change from Period I to Period II was not consistent across treatments. Potassium excretion decreased from Period I to Period II for the Fat treatment while the Starch treatment experienced an increase. Potassium digestibility increased for the Fat treatment from Period I to Period II, but did not change substantially for the Starch treatment.

These results agree with others who reported that the apparent absorption of Mg was not affected by the addition of fat to the diet (McCann et al., 1987; Rich et al., 1981; Davison et al., 1991). Serum levels of Mg were also not affected by fat supplementation in either study (McCann et al., 1987; Rich et al., 1981).

The mean Mg digestibilities found in the current study (48.47% for the Starch treatment and 50.51% for the Fat treatment) are somewhat higher than those reported by McCann and coworkers (1987) which was 33.4% for control horses and 35.3% for those supplemented with 10% corn oil. However, Davison et al. (1991) reported Mg digestibilities between 37.7 and 45.8% for control horses and 43.8 and 47.3% for those on added fat diets. On the other hand, Schryver et al. (1987) calculated the true

absorption of Mg in horses to be between 62 and 67%. Magnesium absorption may vary anywhere from 40-60% on average depending on the source (NRC, 2007).

Comparable studies outlining the effect of fat supplementation on K absorption in the equine species is lacking. However, total tract absorption of K was unaffected by fat supplementation in dairy cattle fed 0, 2.5 or 5% fat (Rahnema et al., 1994). In addition, mean K digestibilities in the current study are in agreement with the range of 61 to 75% observed by previous studies (NRC, 2007). Pagan (1994) estimated true K absorption at 75% while Pagan and Jackson (1991) reported a range of 61 to 65% for apparent K digestibility. The K digestibility results of the present study are consistent with published equine research although reports of an associative effect of fat supplementation on K digestibility in horses are scarce.

See the following page for Table 10.

Table 10. Effects of a high-fat diet (Fat) and high-starch diet (Starch) on daily magnesium and potassium intake, excretion, and digestibility (least squares means \pm SEM)

Response	Treatment	
	Fat (n = 8)	Starch (n = 9)
Period I		
Magnesium intake, g/d	11.75 \pm 0.464	11.72 \pm 0.437
Magnesium excretion, g/d	5.62 \pm 0.309	5.47 \pm 0.291
Magnesium digestibility, %	52.18 \pm 2.12	53.21 \pm 2.00
Period II		
Magnesium intake, g/d	12.00 \pm 0.411	12.60 \pm 0.388
Magnesium excretion, g/d	6.13 \pm 0.350	7.06 \pm 0.330
Magnesium digestibility, %	48.83 \pm 2.65	43.74 \pm 2.50
Period I		
Potassium intake, g/d	66.91 \pm 2.45	60.39 \pm 2.31
Potassium excretion, g/d	23.76 \pm 1.97	22.79 \pm 1.86
Potassium digestibility, %	64.24 \pm 2.70	62.68 \pm 2.54
Period II		
Potassium intake, g/d	68.26 \pm 2.14	64.04 \pm 2.02
Potassium excretion, g/d	20.06 ^a \pm 1.71	25.32 ^b \pm 1.62
Potassium digestibility, %	70.66 ^a \pm 2.19	60.63 ^b \pm 2.06

^{a,b}Means within a row without a common superscript differ ($P < 0.05$).

Parameters of growth - body weight

There was no difference in baseline body weight measurements between horses on the starch diet compared to those on the fat diet. There was no diet x week effect; however, there was a diet effect and a week effect meaning that as time went on, all horses gained weight during the trial.

Perhaps most notable was the fact that there was a difference in body weight between horses on the starch diet and those on the fat diet ($P = 0.023$). Horses fed the starch diet were 5.6 kg heavier on average by the end of the trial than those individuals on the fat diet. This is in contrast to observations by others that cite no differences with respect to body weight between fat-supplemented horses and controls although DE intake was similar between the treatment groups (Orme et al. 1997; Graham-Thiers et al., 2001; Pagan et al., 2002). Orme et al. (1997) found no significant effect of diet on body weight between high-fat versus low-fat supplemented horses during a treatment period of 10 weeks in which the DE intake was calculated to be the same between the two diets. Eaton et al. (1995) also found that while the fat-supplemented horses' body weights remained consistent, the body weights in the control group increased 1.3% on average with diets that were formulated to be isocaloric.

Weight gains were greater for weanlings fed a high-starch vs. an isocaloric low-starch diet (0.81 vs. 0.67 kg/d) while daily DE intakes were similar between the two groups (Ott et al., 2005). Body length measurement was also greater for the weanlings fed the high-starch ration, but no other body measurements were affected by diet in the same study (Ott et al., 2005). At the same time, Hoffman et al. (1999) cited no difference in body weight between starch and sugar supplemented foals, weanlings, and yearlings

versus those supplemented with fat and fiber when fed at equal DE intakes. In contrast, Hoffman et al. (1996) found a decrease in both body weight and condition scores in yearlings fed a corn and molasses based sweet feed as compared to those fed a corn oil and fiber supplement. The authors speculate that since the loss of weight and condition in the sugar and starch group coincided with the appearance of spring pasture growth and both horses were consuming the young immature grasses in preference to the hay offered, the more rapid rate of passage and an excess of rapidly fermentable carbohydrate consumed with the sugar and starch group could have contributed to the loss of weight and condition even though calculated DE intakes were the same between the two groups (Hoffman et al., 1996). Scott et al. (1989) found that yearlings fed 5 and 10% animal fat gained faster in the first 28 days of the trial, but there was no difference in final body weight between fat-supplemented horses and the controls by the end of the 112 day trial.

Digestible energy intake in the aforementioned study was not different between the three treatments. Fat-supplemented horses in the current study actually consumed a greater amount of DE ($P=0.026$) than those on the starch ration (see Table 7); therefore, energy consumption alone cannot explain the difference in final weights. The exact reason(s) for the higher body weights in the Starch group cannot be elucidated without further investigation.

Parameters of growth – skeletal measurements

Effect of diet on skeletal growth parameters are shown in Table 11. There was no significant effect of diet on knee, shoulder, withers or hip measurements. Most growth measurements (knee, shoulder, withers, and hip) showed a significant week effect meaning that as time passed the horses grew.

Findings in the current study are consistent with published literature in that there was no dietary effect on parameters of growth relative to skeletal measurements between fat supplemented horses and controls (Scott et al., 1989; Hoffman et al., 1996; Hoffman et al., 1999; Ott et al., 2005). In a study with foals fed either a sugar and starch ration or a fat and fiber ration from birth to one year of age, no difference was found with respect to growth parameters (Hoffman et al., 1999). Further, Ott et al. (2005) cited that body measurements of heartgirth, wither height, and hip height were unaffected by diet when comparing weanlings fed either a high-starch or low-starch diet; the low-starch diet was made isocaloric with the addition of 5% soybean oil. Hoffman et al. (1996) concluded that there was no difference in overall frame size of weanlings and yearlings on dietary treatments of either corn and sweet feed, or corn oil and fiber. Furthermore, Scott et al. (1989) found no difference in wither or hip height, heartgirth circumference, or rump fat thickness between yearlings fed 5 and 10% fat versus those fed control diets.

See the following page for Table 11.

Table 11. Effect of diet, week, and diet x week interaction on parameters of growth.

	<i>P</i> -values (week 2 thru 8) ¹		
	Diet ²	Week	Diet X Week
Weight	0.023	<0.001	0.457
Withers	0.820	<0.001	0.073
Shoulder	0.101	0.005	0.540
Knee	0.494	0.044	0.840
Hip	0.131	<0.001	0.659
Hock	0.153	0.136	0.982

¹Week 1 was used as a baseline measure

²Fat diet (n=8), starch diet (n=9)

Heart rate

Physiological responses such as heart rate have been well documented in research involving dietary fat supplementation in horses. In the current study there was no significant time ($P=0.739$) or time x diet interaction ($P=0.978$). There was, however, a strong association between resting heart rate and heart rate after exercise ($P=0.008$), and this association was significant for horses on the starch diet ($P=0.002$) but not significant for those on the fat diet ($P=0.722$). That is for horses on the starch diet, higher resting heart rates resulted in higher heart rates during exercise. However, there was no effect of diet on resting heart rate nor was there a difference in heart rate response to a fixed exercise challenge between horses on the high-fat ration versus those on the high-starch ration (Figure 1). Results of the current study are consistent with previous research in horses which indicate no difference in heart rate between fat-supplemented horses and controls (Eaton et al., 1995; Pagan et al., 2002; Lindberg et al., 2006).

To counter these findings, Pagan et al. (1987) reported significantly lower heart rates for fat-supplemented horses during a stepwise exercise test in addition to the recovery phase, but at the same time, found no differences between heart rates for horses on the control and fat diets during a 105 min long, slow work test at 5 m/sec. In addition, Duren et al. (1999) reported decreased heart rates in the first two minutes of a standard exercise test (SET) in fat-supplemented horses but found no significant time x diet interaction with respect to heart rate. Others have also observed that high starch intakes may cause elevated heart rates (MacLeay et al., 1999) along with a greater tendency for excitability (Pagan et al., 1987; MacLeay et al., 1999). In addition, the form of

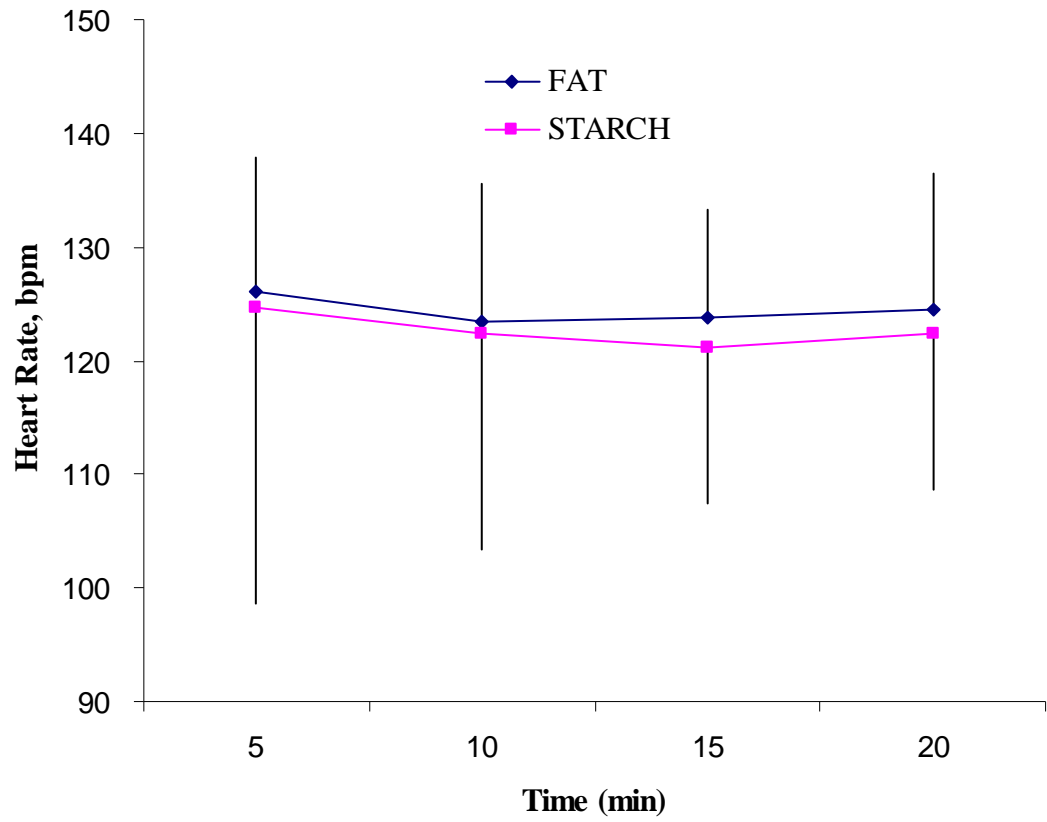
carbohydrate may also cause alterations in heart rate. When barley sugar was substituted for an oats at the 1.0 and 1.5 kg/d level, elevated heart rates resulted during a submaximal exercise test (Jansson et al., 2002).

Some studies found that heart rates recovered more rapidly after exercise when fed a fat-supplemented diet (Meyers et al., 1987; Pagan et al., 1987) but others reported that heart rates in fat-supplemented horses recovered more slowly after repeated exercise bouts even though heart rates at the end of exercise were similar in both the fat-supplemented and the control horses (Webb et al., 1987).

Comparison of studies involving fat supplementation in horses regarding heart rate is difficult due to the tremendous variation in study design such as fat adaptation, level of conditioning, and intensity of the exercise challenge. All of these factors could have an impact on resulting heart rates at rest, during exercise, and post-exercise.

See the following page for Figure 1.

Figure 1. Time effect on exercising heart rates per dietary treatment.



Blood metabolites

Comparisons of baseline measurements (Time 1) for plasma β -hydroxybutyrate (BHB), glucose, lactate (LA), and triglyceride (TG) concentration showed no differences between the two treatment groups. These results are not surprising considering all horses were of similar age, on the same diet, and had similar activity level prior to commencement of the study. Results for post-treatment (Time 2) and post-exercise (Time 3) analysis of BHB, glucose, LA and TG are shown in Table 12. Values for acetoacetate (AcAc) are not shown due difficulties encountered during the analysis.

Blood glucose. There was no effect of diet on plasma concentrations of glucose ($P=0.128$) at Time 2, nor at Time 3 ($P=0.324$) (Table 12).

These results agree with Ott et al. (2005) who reported similar fasting blood glucose measurements between weanlings consuming a low-starch diet and those on a high-starch diet. In addition, Pagan et al. (1995) reported no difference in resting plasma glucose concentration between fat-supplemented horses and controls after an overnight fast in 2 year-old Thoroughbreds.

The lack of a difference in plasma glucose concentration immediately after a slow aerobic test in the present study is also consistent with Pagan et al. (1995) who found no difference in blood glucose during or after a SET when the horses trotted for 30 min at a relatively slow speed of 4-4.5 m/s. When horses in the previous study reached a speed of 11 m/s, the fat-supplemented horses had a higher blood glucose concentration than the controls and it remained elevated both at 15 and 30 minutes post-exercise. Similar results were reported by Taylor et al. (1995) in which fat-supplemented horses had higher blood

glucose levels during a SET to fatigue, but again, no differences were found with respect to resting glucose concentrations. Duren et al. (1999) also reported higher glucose concentrations for fat-supplemented horses 15 and 30 minutes post-exercise following a SET. Exercise intensity in the current study did not reach the intensity of the SET from the previous studies. Therefore, exercise intensity in the current study would have to be greater than the 4 m/s performed to elicit a difference in plasma glucose concentration between the two dietary treatments as the duration and intensity of exercise likely would not have posed major challenges to the blood glucose homeostatic mechanism.

Lactate . There was no effect of diet on plasma concentrations of LA ($P=0.248$) at Time 2 nor at Time 3 ($P=0.850$) (Table 12).

Results from the present study are consistent with previous equine research involving fat supplementation (Greiwe et al., 1989; Oldham et al., 1990; Crandell et al., 1999; Duren et al., 1999; Lindberg et al., 2006) in which no difference was observed in plasma [LA] after an overnight fast.

Results from the present study are consistent with Greiwe et al. (1989) in which plasma lactate concentrations were not affected by treatment when horses were fed 10.5% added dietary fat for 12 weeks. The exercise challenge in the aforementioned study was similar to the current study in that the SET consisted of the horses trotting to a stable heart rate of 160 bpm. Furthermore, plasma lactate concentration did not differ between dietary treatments after exercise while trotting at 4 m/s for those horses fed 0, 5, 10, and 20 % fat in the form of corn oil (Duren et al., 1987). Duren and coworkers (1999) reported that plasma lactate concentrations increased with increasing exercise

intensity in the SET, but were not affected by dietary treatment such as fat supplementation in 3 year old Thoroughbreds. Other studies also reported no difference between fat supplemented horses and controls with respect to plasma lactate concentrations taken at assorted times after exercise of varying intensities (Oldham et al., 1990; Crandell et al., 1999; MacLeay et al., 1999).

In contrast, Eaton et al. (1995) reported higher plasma lactate concentrations 5 minutes post-exercise for fat-supplemented horses. Taylor et al. (1995) also reported blood lactate levels 39% greater in fat-adapted horses than controls at the end of a SET to fatigue which they explained may be due to an increased rate of glycogenolysis coupled with a reduction in pyruvate conversion to acetyl CoA by the pyruvate dehydrogenase complex (PDC). Both Eaton et al. (1995) and Taylor et al. (1995) exercised horses at a higher intensity than that put forth by the horses in the present study.

Lactate is the end product of *anaerobic* metabolism and therefore its concentration in plasma increases during high-intensity exercise with typically the highest concentrations seen 2-10 minutes post-exercise (Pösö et al., 2004). The aerobic exercise challenge in the present study did not elicit a work effort of high enough intensity to produce not only an overall increase in lactate concentrations but also a difference in concentration between the Fat horses versus the Starch horses.

Triglycerides. There was no effect of diet on plasma concentrations of TG at Time 2 ($P=0.921$) or at Time 3 ($P=0.753$) (Table 12).

The findings of the present study reporting no dietary difference in plasma triglyceride (TG) concentration at rest is consistent with some published research

(Crandell et al., 1999; Marchello et al., 2000; Zeyner et al., 2002; Treiber et al., 2005), but inconsistent with other research that found resting levels of plasma TG to be lower in fat-supplemented horses as compared to the controls (Duren et al., 1987; Orme et al., 1997; Geelen et al., 1999; Geelen et al., 2000; Geelen et al., 2001a; Sloet van Oldruitenborgh-Oosterbaan et al., 2002).

Duren et al. (1987) speculates that the higher TG concentrations observed in their low-fat diets may be due to the release of VLDL into circulation caused by the conversion of glucose to TG in the liver. Orme et al. (1997) reported significantly lower resting plasma TG concentration after an overnight fast in aerobically conditioned horses after 9 weeks of fat-supplementation (20% DE as soybean oil = 1.0 g fat per kg body weight); the authors suggest that as a result of conditioning, muscles are better able to utilize the lipoprotein-associated plasma TG because of an increase in the activity of muscle lipoprotein lipase (LPL) causing the reduction in plasma triacylglycerol concentration. The decrease in plasma TG observed by Orme et al. (1997) was associated with a mean 50% increase in plasma total lipase activity following pentosan polysulfate administration (which causes the release of both lipoprotein lipase and hepatic lipase into circulation) as well as an increase in postprandial plasma cholesterol concentration and a significant increase in the activity of muscle citrate synthase. Geelen et al. (1999, 2000) also found an increase in LPL activity along with a decrease in plasma TG in fat-supplemented horses, possibly indicating an increased flux of fatty acids to extra-hepatic tissues according to the authors. Geelen et al. (2001a) speculates that “the higher oxidative ability together with the depressed rate of de novo fatty acid synthesis in liver may contribute to the dietary fat-induced decrease in plasma TAG concentrations in

equines.” The explanations for the lower plasma TG concentration in fat supplemented horses are speculative as not all studies found a link between decreased plasma TG levels and increased LPL activity.

Contrary to the findings mentioned above, Geelen et al. (2001b) reported that fat supplementation had no effect on resting plasma TG concentration after an overnight fast despite fat-induced increases in LPL and hepatic triacylglycerol lipase (HTGL) activities in conjunction with increases in plasma concentrations of total cholesterol, HDL cholesterol, and total phospholipids. Lipoprotein lipase activity was calculated as the difference between total and hepatic lipase activities (Geelen et al., 2001b). Other authors have also found no significant dietary difference in resting plasma TG concentrations between fat supplemented horses and controls (Crandell et al., 1999; Marchello et al., 2000; Zeyner et al., 2002; Treiber et al., 2005). Zeyner et al. (2002) found that the concentration of plasma TG was not influenced by diet type for horses fed a high-starch or high-fat ration for 390 days. At the same time, Treiber et al. (2005) found no difference in resting plasma TG concentration due to diet in starch and sugar supplemented weanlings as compared to those supplemented with fat and fiber. The authors that reported lower resting plasma TG concentrations in fat supplemented horses (Duren et al., 1987; Orme et al., 1997; Geelen et al., 1999; Geelen et al., 2000; Geelen et al., 2001a; Sloet van Oldruitenborgh-Oosterbaan et al., 2002) did not make an attempt to separate the various lipoprotein fractions to quantify exactly where the differences might exist with regard to plasma TG concentration as it is influenced by diet.

Marchello et al. (2000) separated the various lipoprotein fractions of plasma TG for 2 year olds of Quarter Horse and Thoroughbred-type supplemented with either 20%

of their daily DE as rolled corn or corn oil for 10 weeks. The authors reported higher protein concentrations of very low density lipoproteins (VLDL), high density lipoproteins (HDL), and albumin (ALB) fractions along with higher cholesterol in the fat supplemented group versus the control (Marchello et al., 2000). The difference between the two experimental groups in plasma TG content affected primarily the VLDL fractions. For instance, the fat supplemented horses had very high VLDL-TG fractions and low concentrations of the LDL fraction (Marchello et al., 2000). In contrast, the control horses had moderate VLDL fractions without as much of a dramatic decrease in the LDL fraction; HDL-TG values were highly variable. Very low density lipoproteins are thought to contribute as fuel to exercise (Jeukendrup et al., 1998a) as VLDL is the primary lipoprotein responsible for transport of triacylglycerols from the liver to adipose tissue and muscle (Murray et al., 2006). Marchello and others (2000) indicate that the difference is attributed to an assumed increase in LPL activity in the fat-fed horses, just as the authors suggested who reported a decrease in plasma TG content in the fat-supplemented horses (Orme et al., 1997; Geelen et al., 1999; Geelen et al., 2000; Geelen et al., 2001a; Sloet van Oldruitenborgh-Oosterbaan et al., 2002). Although the relationship between fat intake and LPL activity seems to be linear (Geelen et al., 2001b), the physiological significance of the differences observed remains in question.

The findings of the present study reporting no difference in plasma TG concentration immediately following exercise are inconsistent with Duren et al. (1987) who reported lower plasma TG concentrations in those horses fed a diet consisting of 5, 10 or 20% fat as compared to those fed 0% fat. In addition, Sloet van Oldruitenborgh-Oosterbaan and coworkers (2002) found significantly lower plasma TG concentrations

during and after a submaximal SET for those horses on a diet consisting of 11.8% fat as soybean oil. Similar to the current study, Crandell et al. (1999) reported no difference in TG response to exercise across isocaloric dietary treatments consisting of a standard carbohydrate diet, a high fat diet consisting of 15% of the DE as soybean oil, and a diet where 15% of DE was beet pulp. Results of published research in the equine species concerning dietary effects on plasma TG concentration during exercise are inconsistent. Furthermore, variations in study design, level of fat supplementation, and exercise protocols make it difficult to compare results. More investigation is needed to determine dietary effects on plasma TG concentrations at rest as well as during and immediately after exercise.

Acetoacetate. Due to negative values obtained in AcAc analysis, data is thought to be invalid for drawing conclusions.

β -hydroxybutyrate. There was a dietary effect on plasma concentrations of BHB at Time 2 with the starch diet yielding a larger mean ($P=0.036$) than the fat diet, no matter what baseline value was observed. However, there was no effect of diet at Time 3 ($P=0.725$) (Table 12).

Results of the present study indicating a dietary effect on resting plasma BHB concentration after an overnight fast are in agreement with Harkins et al. (1992) who reported a significant increase in resting plasma BHB concentration for those horses on a control diet versus those that were supplemented with 12% of their daily DE as fat. The opposite effect was found in human subjects where dietary carbohydrate restriction

produced an increase in circulating ketone bodies after exercise in highly trained marathon runners (Koeslag et al., 1980). Koeslag et al. (1980) suggested that the critical factor in the development of post-exercise ketonaemia in humans is the carbohydrate status of the body which is perhaps even more important than exercise duration, intensity, or even conditioning level. However, Rose and Sampson (1982) contend that the ketone pathway is relatively unimportant in the horse, but they did not investigate dietary effects. Ribeiro and coworkers (2004) found that there were no significant differences found in plasma BHB concentrations among treatment diets in blood samples taken 4 hours after the last exercise session consisting of 2 minute intervals of walking (1.8 m/s) and trotting (3.2 m/s) for a maximum of 30 minutes. Diets ranged from 7.2% fat and 21.2% starch to 12.7% fat and 3.9% starch (Ribeiro et al., 2004). Furthermore, the type of dietary fat fed to horses appears to have no effect on resting plasma ketone levels as there was no difference in resting plasma BHB concentration taken after an overnight fast between diets supplemented with either saturated palm oil or unsaturated soybean oil (Hallebeek and Beynen, 2002). Post-exercise ketosis in humans has been found to be a function of carbohydrate availability, but more investigation is required to determine the precise dietary mechanism involved in ketonaemia in horses.

Similar studies evaluating the effect of diet on plasma BHB concentration as it relates to exercise in the horse are scarce. The results of the current study finding no difference in BHB concentration pre- and post-exercise are in agreement with Harkins et al. (1992). Harkins and coworkers (1992) reported no difference in plasma BHB concentration at 2 and 4 minutes after a 1600 m race between fat supplemented horses and controls; however, BHB concentration was significantly higher for control horses at

8 and 16 minutes post-exercise. Other researchers evaluating the effect of exercise on plasma BHB concentrations found no difference in BHB concentration when comparing pre- and post-exercise values (Dybdal et al., 1980; Rose and Sampson, 1982). Rose and Sampson (1982) reported no change in plasma BHB or AcAc concentration when comparing samples taken before an 80 km endurance ride (246 m/min = 4.1 m/s) and those taken immediately after completion of the event. However, they did find a significant increase in plasma BHB concentration at 30 minutes post-exercise. The authors speculate that the small but significant increase in BHB at 30 min post exercise may be due to high circulating concentrations of non-esterified fatty acids (NEFA) so that mobilization exceeded utilization, resulting in an increase in hepatic ketoacid production. Lucke and Hall (1980) reported a significant increase in total ketone (AcAc + BHB) concentration 5 minutes after an 80 km endurance ride as well as 60 minutes after the ride. The results from these studies suggest that sampling more than 4 minutes after cessation of exercise is necessary to detect a change in circulating BHB concentration.

On the other hand, Rose et al. (1980) found a significant decrease in plasma BHB concentration immediately after the roads and tracks and steeplechase (650 m/min = 10.8 m/s) phases of a three-day event competition. The mean speed of the roads and tracks phase was 240 m/min (4 m/s) which was close to the speed elicited in the exercise challenge in the current study. Interestingly enough, there was no change in BHB concentration immediately after the cross country phase of the event in which horses competed at a mean speed of 490 m/min (8.17 m/s) (Rose et al., 1980). The reason for the decrease in BHB concentration 30 seconds upon completion of the roads and tracks and steeplechase phases escapes the authors, but they speculate that it may be due to the

difference in NEFA metabolism between the horse and man. With increased fat mobilization one would expect a concurrent increase in ketoacids, according to the authors (Rose et al., 1980). Lucke and Hall, (1980) reported an increase in lipolysis signified by a rise in both free fatty acids and glycerol levels but it was not associated with a parallel increase in circulating ketones. The authors suspect that the results indicate that it is unlikely that the ketones contribute as metabolic substrates to any degree and “post-exercise ketosis” is not a feature of long distance riding (Lucke and Hall, 1980). Rose and Sampson (1982) contend that ketosis may not be a feature of prolonged exercise in the horse due to the ability of the liver to maintain glycogenolysis and therefore the supply of carbohydrate for the citric acid cycle. Dybdal and colleagues (1980) suggest that a difference in circulating ketones was not demonstrated when comparing samples taken before and after a 160 km endurance ride due to the possibility that exercise duration was insufficient or more likely that ketone mobilization matched utilization. Exercise duration in the present study was likely insufficient to induce a difference in circulating ketones. Likewise, sampling protocol would also need to be evaluated.

See the following page for Table 12.

Table 12. Least squares means, standard errors (in parenthesis), and observed significance levels of effects on blood metabolites by treatment

Metabolite	Treatment				P Value	
	Diet ¹		Post-exercise ²			
	Fat (n = 8)	Starch (n = 9)	Fat (n = 7) ³	Starch (n = 9)	Diet	Post - exercise
β-hydroxybutyrate, mg/dL	1.899 ^{4a} (0.177)	2.464 ^{4b} (0.167)	1.994 ⁵ (0.189)	2.061 ⁵ (0.190)	0.036	0.725
Glucose, mg/dL	92.494 (1.674)	88.783 (1.578)	68.607 (4.775)	75.122 (4.211)	0.128	0.324
Lactate, mg/dL	5.206 (0.223)	4.862 (0.214)	5.721 (0.550)	5.833 (0.509)	0.248	0.850
Triglycerides, mg/dL	21.331 (1.807)	21.583 (1.704)	18.898 (3.742)	20.074 (3.503)	0.921	0.753

¹Time 1 (baseline/pre-treatment) to Time 2 (post-treatment/pre-exercise) comparison

²Time 2 (post-treatment/pre-exercise) to Time 3 (post-exercise) comparison

³Fat group has an n of 7 as one horse did not complete the exercise challenge

⁴Least squares means adjusted for the average BHB value of the baseline (time 1) covariate (2.58)

⁵Least squares means adjusted for the average BHB value of the pre-exercise (time 2) covariate (2.16)

^{a,b}Within a row and treatment, means that do not have a common superscript differ ($P < 0.05$)

Hormones

Comparisons of baseline measurements (Time 1) for hormone concentration of insulin, leptin, and adiponectin in equine plasma showed no differences between the two treatment groups. These results were expected considering all horses were of similar age, on the same diet, and had similar activity level prior to commencement of the study.

Insulin. There was no effect of diet on plasma concentrations of insulin at Time 2 ($P = 0.369$) nor at Time 3 ($P = 0.718$) (Table 13).

Insulin is responsible for maintenance of blood glucose levels (Thornton, 1985); however, it is suppressed during exercise (Thornton, 1985; Freestone et al., 1991; Gordon et al., 2007b) at a threshold of 50% VO_{2max} (Thornton, 1985). Working muscles can take up glucose without insulin and the horse is still able to maintain blood glucose levels through increased gluconeogenesis during exercise (Thornton, 1985). An increase in insulin will inhibit the release of FFA from adipose tissue by inhibiting the activity of hormone sensitive lipase resulting in a decline in circulating plasma free fatty acids (Murray et al., 2006). In addition, insulin enhances lipogenesis, in which glucose is converted into fatty acids, and the synthesis of acylglycerol (Murray et al., 2006). Since insulin facilitates glucose uptake by the cells, it is a promoter of glycogenesis and an inhibitor of gluconeogenesis; hence, insulin is vital to the recovery phase of exercise for glycogen repletion (Thornton, 1985; Pratt et al., 2007).

Acute changes in postprandial insulin concentrations have been well documented in horses (Pagan et al., 1995; Duren et al., 1999; Ropp et al., 2003; Gordon and McKeever, 2006). Plasma insulin peaked between 60 and 90 min both after consumption

of grain and in an intravenous dextrose test - insulin peaked at 90min post-infusion while glucose peaked at 5 min (Gordon and McKeever, 2006). In both tests, insulin was positively correlated with glucose and negatively correlated with ghrelin, cortisol, and adiponectin (Gordon and McKeever, 2006). Hoffman et al. (2003) reported no effect of diet during a modified frequent sampling i.v. glucose tolerance test (FSIGT) on endogenous insulin response in starch and sugar (SS) supplemented geldings versus those supplemented with fat and fiber (FF) for 8 weeks. Similar findings were reported by Treiber et al. (2005) in which SS weanlings had greater insulin concentrations during the FSIGT as compared to the FF weanlings. Also, the SS weanlings showed a propensity for insulin resistance as indicated by lower insulin sensitivity versus the FF weanlings. Although insulin concentrations were not statistically different, there was a trend for higher insulin concentrations in the SS foals (Treiber et al., 2005). There is a trend for higher carbohydrate meals to elicit greater increases in insulin concentrations than those meals consisting predominately of fat and fiber.

Time of feeding will also effect insulin concentrations. Ropp et al. (2003) sampled weanlings for up to 6 hours after feeding and found that by day 60, the insulin response to feeding was less over time in fat supplemented horses versus the starch supplemented horses. Horses fed 3 hours prior to exercise as compared to those fed 8 hours prior to exercise or those subjected to an overnight fast, experienced elevated plasma glucose and insulin at rest regardless of dietary treatment (Duren et al., 1999). Furthermore, plasma glucose was decreased during exercise and fat oxidation was suppressed as indicated by lower NEFA concentrations. Fat supplementation did not

affect plasma insulin concentrations at rest, but did have lower plasma insulin responses during exercise as well as at 15 min, but not 30 min post-exercise (Duren et al., 1999).

Our results are consistent with Pagan et al. (1995) who reported no difference with respect to diet on resting plasma insulin concentrations in samples taken 4 hours after feeding in control versus fat-supplemented 2 year-old Thoroughbreds in training. In addition, our post-exercise results are consistent with Pagan et al. (1995) who found no difference due to dietary treatment on plasma insulin levels. Exercise performed by both Duren et al. (1999) and Pagan et al. (1995) was more strenuous than that performed in the present study (20 min of trotting at 4.0 m/s). Therefore, there was no effect of diet on insulin concentrations post-exercise likely because the exercise challenge did not exceed 50% VO_{2max} . Additionally, samples in the current study were collected following an overnight fast; therefore, meal-dependent alterations in plasma insulin concentrations would not have been evident. Any difference in insulin concentrations would have been due to long-term dietary effect of feeding fat or starch rich diets to the experimental horses over the 8 week period.

Leptin. There was a significant effect of diet on plasma concentration of leptin at Time 2 ($P = 0.010$), but only when baseline values were 1.4 and larger. There was no dietary effect ($P = 0.750$) on plasma leptin concentrations at Time 3 (Table 13).

Leptin is a protein product of the obese gene (Friedman and Halaas, 1998) and the greater amount of adipose tissue, the greater amount of leptin produced (Kearns et al., 2006). Resting leptin levels in horses are positively correlated with body condition score

(Gordon et al., 2007a) as well as % fat mass and % body fat (Kearns et al., 2006; Gordon et al., 2007a) in horses and in humans (Weltman et al., 2000).

The current study is the first according to our knowledge to examine the role of dietary fat and starch on plasma leptin concentrations in the equine species both at rest and after exercise. Dietary effects have been studied in other species to investigate obesity and the associated pathologies. Plasma leptin concentration decreased along with leptin mRNA expression in adipose tissue in insulin resistant rats following 3 weeks of fat supplementation with 10% marine oils plus 18 % saturated fat versus 28% saturated fat (Ukropec et al., 2003). Body weights were lower in the marine oil supplemented rats in the same study (Ukropec et al., 2003). Lee et al. (2006) reported no difference in plasma leptin concentration between standard chow fed rats and those supplemented with 17.6% lard for 6 weeks despite higher energy intake and body weight gain in the latter. On the other hand, Zhou et al. (2008) reported elevated plasma leptin levels in high-fat fed rats which also had higher body weights and fat contents. The fat-fed rats in the previously mentioned study did develop insulin resistance denoted by significantly higher blood glucose and insulin levels after feeding as compared to the rats on the basic/control diet (Zhou et al., 2008).

Studies in humans confirm that high-fat, low-carbohydrate meals, which produce smaller insulin and glucose responses, decrease leptin concentration (and could contribute to weight gain) while low-fat, high-carbohydrate meals increase leptin concentration (Havel et al., 1999). The subjects in Havel et al. (1999) were not diabetic and also not subjected to insulin resistance. Evans et al. (2001) reported a decrease in plasma leptin concentration as a response to both oral and intravenous fat loads in human

subjects with no statistical differences between the two. On the other hand, leptin increased slightly with a mixed meal. Arterial and venous leptin concentrations continued to increase for up to 5 hours after consumption of a high carbohydrate meal in another [human] study (Coppack et al., 1998).

There was a decrease in plasma leptin concentration when horses were subjected to an oral grain challenge while leptin increased in response to an intravenous dextrose solution test (Gordon and McKeever, 2006). There appears to be consistency among the three species pertaining to plasma leptin concentrations. First, higher body weight and fat contents increase leptin production. Next, meals with a greater fat content and thus a smaller glucose and insulin response tend to decrease leptin levels while mixed meals or those high in carbohydrates increase plasma leptin levels.

Results in the present study show that it was the Starch fed horses that ultimately had a small, but statistically significant increase in body weight by the end of the 8 week trial, but fat mass was not investigated. In addition, blood samples were not drawn after a meal feeding, but rather were collected after an overnight fast for Time 1 and Time 2, as well as immediately post-exercise (Time 3). Furthermore, horses in the current study displayed no difference with respect to diet in concentration of glucose or insulin.

Plasma leptin concentrations have been found not to change during or immediately after exercise in humans (Essig et al., 2000; Hulver and Houmard, 2003; Ferguson et al., 2004) and horses (Gordon et al., 2007b). It appears that sampling 24 to 48 hours post-exercise may be necessary to see changes in plasma leptin concentrations. Studies involving leptin concentration analysis pertaining to exercise in humans indicate that a sufficient energy deficit must occur in order to decrease leptin levels (Hulver and

Houmard, 2003). For instance, while short term exercise (< 60 min) of various intensities do not cause a shift in leptin levels (Weltman et al., 2000), one study indicated that long term exercise (> 60 min) may produce enough of a kilocalorie deficit to produce a disruption in long-term energy balance (Karamouzis et al., 2002). There was no alteration in plasma leptin levels and body composition remained unaltered when moderate exercise was performed by human subjects over three, 3 day periods even though a slightly negative energy balance was reported (Dirlewanger et al., 1999). On the other hand, a prolonged exercise effort such as a marathon swim produces enough of a negative energy balance to decrease leptin concentration and therefore increase food intake to maintain energy homeostasis (Karamouzis et al., 2002). A recent study in horses has shown that short-term, high-intensity exercise in horses decreases plasma leptin concentration 24 hours post-exercise, signaling alterations in energy intake (Gordon et al., 2007b). Results of studies in humans and horses indicate that energy homeostasis must be disrupted before decreases in leptin will occur.

The sampling protocol on the current study did not allow for post-prandial differences to be seen with respect to diet and plasma leptin concentration. Furthermore, the exercise challenge likely did not solicit enough of an energy deficit to produce a difference in plasma leptin concentration with respect to diet. In addition, Time 3 samples were taken immediately post-exercise which would not have allowed sufficient time to alter leptin concentrations should there have been any.

Adiponectin. There was no difference due to diet in plasma adiponectin concentration at Time 2 ($P=0.244$) nor at Time 3 ($P=0.076$) (Table 13).

Adiponectin and leptin are two hormones related to energy homeostasis. The inverse of leptin, resting levels of plasma adiponectin are negatively correlated with body condition score, body weight, and % fat in horses (Kearns et al., 2006; Gordon et al., 2007a) as well as % fat mass in humans (Matsubara et al., 2002; Tschritter et al., 2003). Brichard et al. (2003) hypothesized that fat mass may apply a negative feedback on its own adiponectin production. Its role in metabolism is related to the regulation of glucose, insulin and adipocyte metabolism (Tsao et al., 2002); therefore, high adiponectin levels may also indicate increased insulin sensitivity (Maeda et al., 2001; Brichard et al., 2003; Tschritter et al., 2003). Furthermore, adiponectin has been found to be negatively correlated with leptin concentrations in humans (Matsubara et al., 2002) and horses (Kearns et al., 2006; Gordon et al., 2007a) and positively correlated with ghrelin in horses (Gordon et al., 2007a). Therefore, it is no surprise that plasma adiponectin levels were found to be greater in fit horses versus unfit horses (Gordon et al., 2007a).

The present study is the first according to our knowledge that has investigated the relationship between plasma adiponectin concentrations and diet in the equine species. However, when horses were subjected to either an oral grain challenge or an intravenous dextrose solution test, there was no change in adiponectin levels (Gordon and McKeever, 2006). The type of fat consumed was found to not alter plasma adiponectin in healthy overweight human subjects, but adiponectin significantly increased as a result of weight loss (Kratz et al., 2008). Adiponectin mRNA levels increased in fat-fed rats supplemented with conjugated linoleic acid (CLA) which also corresponded with a reduction in body weights and fat contents (Zhou et al., 2008). It is possible that adiponectin levels are more closely associated with body composition than diet.

Adiponectin concentrations have been found to not be altered with respect to exercise in both horses (Gordon et al., 2007b) and humans (Kraemer et al., 2003; Ferguson et al., 2004). It is suggested that repeated exercise bouts may be needed to see changes on plasma adiponectin concentrations due to exercise, at least in overweight [human] males (Kriketos et al., 2004). Adiponectin was found to be positively correlated with insulin sensitivity in humans (Brichard et al., 2003) and exercise has been found to increase insulin sensitivity in horses (Malinowski et al., 2002; Powell et al., 2002) as well as humans (Borghouts and Keizer, 2000; Ferguson et al., 2004). Insulin sensitivity was improved by short-term, low-intensity exercise alone despite lack of change in body weight or body fat percentage in obese mares, but insulin sensitivity was still greater for lean mares (Powell et al., 2002). Insulin sensitivity resulting from exercise in humans did not alter plasma adiponectin concentrations (Kraemer et al., 2003; Ferguson et al., 2004). However, Tschritter et al. (2003) reported an increased RQ indicating a shift from lipid oxidation to carbohydrate metabolism during a hyperinsulinemic-euglycemic clamp; there was a greater proportion of glucose disposed of under the action of insulin in combination with high adiponectin concentrations in human subjects. The relationship between insulin sensitivity due to exercise and alterations in adiponectin concentrations in both horses and humans need further clarification.

In the present study, 8 weeks of fat supplementation did not produce a significant alteration in plasma adiponectin levels when compared to a traditional high starch ration either at rest or after an aerobic exercise challenge. It can possibly be concluded that a shift in body composition would need to occur or repeated exercise bouts employed in order to see a difference between dietary treatments.

Table 13. Least squares means, standard errors (in parenthesis), and observed significance levels of effects on plasma hormone concentration by treatment

Hormone	Treatment				P Value	
	Diet ¹		Post-exercise ²			
	Fat (n = 7) ³	Starch (n = 9)	Fat (n = 7)	Starch (n = 9)	Diet	Post - exercise
Insulin, μIU/mL	1.984 (0.173)	1.850 (0.165)	2.834 (0.625)	2.683 (0.608)	0.369	0.718
Leptin, ng/mL HE	2.245 ⁴ (0.159)	1.520 ⁴ (0.175)	2.025 ⁵ (0.255)	1.902 ⁵ (0.217)	0.010	0.750
Adiponectin, ng/mL ⁶	6.308 (1.957)	8.539 (1.611)	8.790 (1.009)	7.363 (0.978)	0.244	0.076

¹Time 1 (baseline/pre-treatment) to Time 2 (post-treatment/pre-exercise) comparison

²Time 2 (post-treatment/pre-exercise) to Time 3 (post-exercise) comparison

³Fat group has an n of 7 as one horse did not complete the exercise challenge

⁴Least squares means adjusted for the leptin baseline value of 1.40

⁵Least squares means adjusted for the average leptin value of the pre-exercise (time 2) covariate (1.76)

⁶Number analyzed for adiponectin inconsistent across treatments: Time 1 - Starch n=6, Fat n=6; Time 2 - Starch n=7, Fat n=3; Time 3 - Starch n=8, Fat n=6

Substrate Transporters

Comparisons of baseline measurements (Time 1) for whole muscle expression of fatty acid translocase/CD36 (FAT/CD36), glucose transporter 4 (GLUT4), monocarboxylate transporter 1 (MCT1) and monocarboxylate transporter 4 (MCT4) in equine skeletal muscle showed no differences between the two treatment groups. No differences were expected considering all horses were on the same diet and had similar activity level prior to commencement of the study.

FAT/CD36. There was an effect of diet on whole muscle expression of FAT/CD36 at Time 2 ($P = 0.006$); however, there was no diet effect on post-exercise scores (Time 3) ($P = 0.273$) (Table 14). Our findings of increased expression of FAT/CD36 protein in conjunction with a high-fat diet but not with endurance conditioning, are consistent with those in human skeletal muscle in which a fat-rich diet increased FAT/CD36 expression (Cameron-Smith et al., 2003; Roepstorff et al., 2004a), but endurance conditioning does not amplify FAT/CD36 protein (Kiens et al., 2004; Pelsers et al., 2007). In contrast, a single bout of exercise (Roepstorff et al., 2004b), or even short-term training (Tunstall et al., 2002; Pelsers et al., 2008) increased FAT/CD36 protein expression slightly but there was no difference in FAT/CD36 content in the vastus lateralis muscle when comparing endurance trained and untrained human subjects (Kiens et al., 2004; Pelsers et al., 2007). The increased expression of FAT/CD36 with short-term training or even a single exercise bout may have more to do with the effect of the last exercise bout and not the training itself as biopsies in Tunstall et al. (2002) were taken within 24 hours of the last exercise session (Kiens et al., 2004). Kiens (2006)

suggests that increased FAT/CD36 expression is an early adaptation to increased muscle activity and that it may fade with sustained activity, such as with exercise conditioning.

Increased expression of FAT/CD36 due to diet was also evident in rodent skeletal muscle. While whole muscle FAT/CD36 increased 40% over controls, sarcolemmal membrane-associated FAT/CD36 was increased 56% in the high-fat fed rats versus controls (Smith et al., 2007). Likewise, chronic stimulation (7 days) of the hind limb rat muscles resulted in the up-regulation in total amount of FAT/CD36 present in the total homogenate and in the plasma membrane fraction of skeletal muscle along with an increase in the mitochondrial fraction as compared to control muscles (Campbell et al., 2004). Acute stimulation, however, for 30 min in the same study did not increase the total amount (expression) of FAT/CD36, but did increase amount in the plasma membrane fraction signifying that FAT/CD36 was translocated within the cell.

Research with respect to FAT/CD36 protein expression in equine skeletal muscle is limited. However, van Dam et al. (2004) did quantify the expression of FAT/CD36 protein in relation to fiber types. Their research found that there was a strong expression of FAT/CD36 protein at the sarcolemma of all equine skeletal muscle fibers types from three different muscle groups (triceps brachii, pectoralis descendens, and vastus lateralis muscles) most likely positioned near capillaries to aid in the uptake of LCFA from the plasma. A low intracellular expression of the protein was observed in type 1 fibers, but no intracellular FAT/CD36 protein expression was observed in type 2 fibers in the same study. The current study did not separate the sarcolemma and intracellular FAT/CD36 protein fractions, rather the homogenate consisted of cytosolic fractions from the middle gluteal muscle. Sampling procedure was such that a mixture of both type 1 and type 2

fibers were extracted although muscle fiber typing was not performed in the current study; the superficial portion of the muscle is characterized as being more glycolytic while the gluteus medius becomes more aerobic with increasing sample depth (Kline and Bechtel, 1988; Serrano et al., 2000). Diet and/or exercise were not factors in van Dam et al.'s (2004) research and information on the relationship of FAT/CD36 protein expression with respect to diet and exercise in the equine species is lacking.

Similar findings with respect to fiber type expression of FAT/CD36 occur with rat muscle. Campbell et al. (2004) found that expression of FAT/CD36 was lower in white gastrocnemius (low oxidative muscle fibers) (30.9% lower in subsarcolemmal (SS) and 36.4% lower intermyofibrillar (IMF)) as compared to red gastrocnemius muscle (high oxidative fibers) from female Sprague-Dawley rats.

The current study is the first to determine the effect of diet on FAT/CD36 protein expression in whole muscle in the equine species according to our knowledge. The fact that feeding a high-fat diet as compared to a traditional high-starch diet can vastly increase FAT/CD36 equine skeletal muscle protein expression could be important with respect to increased fatty acid oxidation during aerobic exercise - it could be ascertained that an enhanced amount of FAT/CD36 would be of benefit for clearance of LCFA.

GLUT4. There was no effect of diet on whole muscle expression of GLUT4 at Time 2 ($P = 0.687$) nor at Time 3 ($P = 0.375$) (Table 14).

Our findings are similar to those found in rats with regard to diet and exercise. In rats subjected to 8 weeks of either a high-carbohydrate or high-fat diet, GLUT4 protein expression in both the soleus and extensor digitorum longus (EDL) muscles was similar

with respect to diet in non-conditioned rats; sedentary rats fed a high-fat did not undergo a reduction in skeletal muscle GLUT4 protein (Lee et al., 2002). On the other hand, Kahn and Pedersen (1993) reported a 34% decrease in GLUT4 protein expression along with a 47% decrease in GLUT4 mRNA also in rat skeletal muscle irrespective of fiber type in fat-fed rats as compared to control animals. However, the fat-fed rats in Kahn and Pedersen (1993) were obese and subjected to insulin-resistance and insulin stimulates the rapid uptake of glucose into muscle cells via the GLUT4 transporter (Kahn, 1994). Further, while GLUT4 mRNA expression differed with respect to muscle type (60% higher relative expression in gastrocnemius (type 2 fibers) as compared to soleus (type I fibers)) expression was not altered by dietary treatment in rats fed an isocaloric-sucrose or hypercaloric-fat diet (Ferrer-Martínez et al., 2006). Exercise training resulted in an increase in GLUT4 protein irrespective of diet in the rat soleus muscle, but in the EDL muscle, training-induced increases in GLUT4 protein expression was more evident in the carbohydrate-fed rats (30% increase) than in the fat-fed rats (21% increase) (Lee et al., 2002). The authors suggest that both mRNA and protein expression is controlled independently by exercise and diet (Lee et al., 2002; Ferrer-Martínez et al., 2006).

The GLUT4 protein expression in equine skeletal muscle appears to be fiber type selective. Van Dam and colleagues (2004) reported that type 1 and 2A fibers had a low expression of GLUT4 compared to type 2B fibers in all 3 muscle types (triceps brachii, pectoralis descendens, and vastus lateralis muscles). Research regarding GLUT4 expression in horses has primarily focused on glycogen repletion following exercise. The slower rate of glycogen resynthesis in horses relative to other species after exercise may be due to the lack of increased GLUT4 expression post-exercise (McCutcheon et al.,

2002; Jose-Cunilleras et al., 2005; Pratt et al., 2007). In addition, ingestion of post-exercise carbohydrates as starch-rich meals did not enhance GLUT4 gene expression in muscle compared with isocaloric fiber-rich meals or feed withholding after 3 consecutive days of glycogen depleting exercise (Jose-Cunilleras et al., 2005). As insulin contributes to activation of GLUT4, the low-insulin status brought about by exercise does not favor glycogen resynthesis in horses (Pösö et al., 2004).

Conditioning appears to have little effect on GLUT4 expression in horses; conditioning itself induces a decrease in glucose utilization (McCutcheon et al., 2002). While six weeks of aerobic and anaerobic conditioning increased GLUT4 protein content 2 to 3-fold in equine whole muscle specimens taken post-exercise (4 cm deep from the middle gluteal), GLUT4 content did not differ from pre-exercise samples, regardless of training state (McCutcheon et al., 2002). Thus, there was no change before or after training in GLUT4 protein content with exercise (McCutcheon et al., 2002). In conditioned horses after a single bout of exercise, mean muscle GLUT4 content did not differ between exercised vs. control horses (Pratt et al., 2007). In contrast, Kristiansen et al. (2000) reported that both GLUT4 protein expression and glucose uptake is enhanced by training in humans. The disparity between GLUT4 protein expression and glucose uptake between the two species, equine and human, may help to explain the differences in glycogen repletion following exercise.

Many factors can influence the expression and localization of the GLUT4 transporter, not the least of which could be diet. While it would be easy to presume that the horses in our study on the high-carbohydrate diet would have a greater amount of GLUT4 protein expression, studies in rats have suggested that insulin status may have

more of an effect on GLUT4 expression than diet (Kahn and Pederson, 1993; Lee et al., 2002). In the current study, there was no dietary effect on plasma insulin concentrations between the two treatment groups which could help explain the lack of difference in GLUT4 protein expression between the two treatments. However, research within this area of equine skeletal muscle is lacking, thus making comparisons difficult.

Homogenates were prepared to extract cytosolic components from whole muscle samples in the current study. Therefore, transporters upregulated to the sarcolemmal membrane during exercise would not be expected to be present in the post-exercise protein fractions and a decline in transporter quantity post-exercise was expected to occur. In addition, the time between cessation of exercise and biopsy procedure was perhaps long enough that reinternalization of transporters back into the cytosol occurred, possibly due to the deactivation of AMPK.

Muscle phosphagen pool decreased by exercise, electrically stimulated contraction, or chronically by feeding β -guanidinopropionic acid (β -GPA), results in a phosphorylation (activation) of AMPK which increases the total muscle content as well as the translocation of GLUT4 and FAT/CD36 to the sarcolemma as a response to the depleted energy charge in the cell (Pandke et al., 2008). Beta-guanidinopropionic acid also increased insulin-stimulated glucose transport in rat soleus muscle (Pandke et al., 2008). Likewise, if sufficient time had passed between cessation of exercise and the biopsy, then AMPK would have been deactivated by the sufficiency of energy (ATP in relation to AMP) and reinternalization of the transporters from the sarcolemma to the cytosol could have occurred. During prolonged exercise, however, "AMPK signaling is not a key regulatory system of muscle substrate combustion during prolonged exercise

and its activation by phosphorylation is not sufficient to maintain an elevated acetyl-CoA carboxylase- β (ACC β) Ser²²¹ phosphorylation during prolonged exercise” (Wojtaszewski et al., 2002).

MCT1. There was no effect of diet on whole muscle expression of MCT1 at Time 2 ($P = 0.517$) nor at Time 3 ($P = 0.852$) (Table 14).

To our knowledge, this is the first study to evaluate the effect of diet on MCT1 protein expression in equine skeletal muscle; therefore, comparison between various studies was difficult. A ketogenic diet was found to increase the expression of MCT1 in the brain of rats (Leino et al., 2001); however, no published information was found with regard to dietary effect of MCT1 expression in skeletal muscle no matter what the species.

Monocarboxylate transporter 1 is expressed in skeletal muscle as well as red blood cells (Juel and Halestrap, 1999; Dubouchaud et al., 2000; Koho et al., 2002) and the MCT isoform profile in equine muscle was found to be similar to that in man (Koho et al., 2006). In general, MCT1 is more widely expressed in oxidative fibers and is positively correlated with the percentage of oxidative fibers (Bonen et al., 2000) whereas MCT4 is more abundant in glycolytic fibers. Minor differences in MCT1 isoform exist between type 1 and type 2 fibers in human skeletal muscle (Pilegaard et al., 1999b) whereas in rats, MCT1 is highly related to red oxidative muscles and almost undetectable in glycolytic white muscles (McCullagh et al., 1996; Kobayashi, 2004). Monocarboxylate transporters are responsible for enabling the transport of not only lactate, but also pyruvate and the ketone bodies acetoacetate, β -hydroxybutyrate, and acetate (Juel and

Halestrap, 1999; Enerson and Drewes, 2003). Immunohistochemical analysis of rat skeletal muscle reveals that MCT1 is present in the cell plasma membranes of all type 1 fibers, coordinating with a relatively high uptake of LA into these cells, whereas MCT4 localization occurs in a high percentage of type 2B fibers which reflects the need for the LA produced by muscle contraction to be actively transported out of the cells; both MCT1 and MCT4 are detected on plasma membranes of nearly all type 2A fibers (Kobayashi, 2004).

Skeletal muscle is the main producer of lactic acid in the body due to high rates of glycolysis; lactic acid can be take up by skeletal muscle and heart and used as respiratory fuel (Juel and Halestrap, 1999). MCT1 is responsible for the influx of lactate into skeletal muscle so that it may be oxidized as fuel (McCullagh et al., 1996; Juel and Halestrap, 1999; Dubouchaud et al., 2000). Results from our study conclude that there was no difference in plasma lactate (LA) concentration due to diet before or after exercise. Since blood lactate removal ability is positively correlated with MCT1 protein expression but not MCT4 after a 1-min all-out exercise test in humans (Thomas et al., 2005), it is not unreasonable to expect that MCT1 expression in our study would not be different post-exercise since the exercise intensity performed did not elicit a rise in plasma lactate concentration.

Human subjects that were well-trained, either from sprint training or endurance training, had significantly higher MCT1 expression as compared to less-trained and/or untrained subjects (Pilegaard et al., 1999a; Dubouchaud et al., 2000; Thomas et al., 2005; Bickham et al., 2006). Furthermore, Thomas et al. (2005) found a negative relationship between serum LA concentration and MCT1 and MCT4 contents at the end of

supramaximal exercise as a function of training status; there existed lower [LA] in the best conditioned subjects. Electrically stimulated muscle contraction reduced sarcolemmal MCT1 protein expression by 10% and MCT4 content by 25% in the hindlimb muscles of the rat; all of which were statistically significant (Tonouchi et al., 2002). Monocarboxylate transporter 1 expression appears to not only be species and fiber-type specific, but adding to the inconsistency between studies is the observation that protein fractions evaluated are not always known with absolute certainty. For instance, it appears that Thomas et al. (2005) and Bickham et al. (2006) are using total membranes to determine their MCT protein content, while Tonouchi et al. (2002) is using sarcolemmal fractions and total crude homogenates were used in Pilegaard et al. (1999a) research, but without more detail, it is difficult to determine the exact protein fraction used. More credit should be given to Dubouchaud et al. (2000) as this group of researchers took the time to remove RBC as well as divide protein fractions into total muscle, sarcolemmal, and mitochondrial protein fractions from their muscle samples.

Monocarboxylate transporter 1 is highly related to the oxidative capacities of the cell and activities that increase the mitochondrial density of the muscle cell will also increase the expression of MCT1 in [human] muscle due to its insertion into both sarcolemmal and mitochondrial membranes (Dubouchaud et al., 2000).

Monocarboxylate transporter 1 protein content in human vastus lateralis muscle correlated with citrate synthetase (CS) activity and this reflects an increase in muscle mitochondrial mass due to [endurance] training (Dubouchaud et al., 2000). Furthermore, MCT1 expression in the skeletal muscle of rats is also correlated with CS activity and is

highly related to oxidative capacities of skeletal muscle and the capacities (higher in red oxidative muscles) of the muscle to take up lactate into the cell (McCullagh et al., 1996).

The results of the current study indicate that diet does not play a role in the expression of MCT1 protein and although both MCT1 and MCT4 protein expression can be rapidly upregulated by a single exercise session (Coles et al., 2004), it may be more likely that the concentration of metabolites such as the ketone bodies acetoacetate, β -hydroxybutyrate and acetate, along with lactate contribute to the upregulation of the monocarboxylate transporters since they are responsible for enabling the transport of not only these metabolites, but pyruvate as well (Juel and Halestrap, 1999; Enerson and Drewes, 2003). It is likely that endurance conditioning in horses could produce an increase in sarcolemmal and mitochondrial MCT1 protein content based upon observations in the human and the rat, but only the cytosolic fractions of the myocyte were assayed in the present study. More research would need to take place in which the various protein fractions of the muscle cell are separated and assayed for MCT1 protein content.

MCT4. There was no effect of diet on whole muscle expression of MCT4 at Time 2 ($P = 0.833$) nor at Time 3 ($P = 0.656$) (Table 14).

As with MCT1, to our knowledge, this is the first study to evaluate the effect of diet on MCT4 protein expression in equine skeletal muscle. Monocarboxylate transporter 4 present in all muscle fibers, but in lower concentrations in oxidative fibers; therefore, it is the primary isoform in glycolytic fibers as MCT1 exists primarily in oxidative fibers (Juel and Halestrap, 1999; Kobayashi, 2004) and it is positively correlated with the

percentage of fast-twitch, glycolytic fibers (Bonen et al., 2000). The MCT 4 isoform is clearly more abundant in type 2 fibers in human skeletal muscle, but interindividual variation is high (Pilegaard et al., 1999b). As is the case with MCT1, the MCT4 isoform profile in horses is similar to that in human and rat skeletal muscle (Koho et al., 2006).

There exists a large variation in published research with regard to conditioning effects on MCT4 protein expression, indicating that MCT4 is less sensitive to conditioning effects than MCT1. High-intensity exercise significantly increased protein expression of MCT4 in one study after 8 weeks of high-intensity one-legged knee extensor exercise; the average contents of MCT1 and MCT4 protein in the trained muscle were 70 and 33% higher respectively, than in the untrained muscle (Pilegaard et al., 1999a). However, a similar study using the same exercise for the same conditioning duration, failed to find a statistically significant increase in MCT4 protein expression although a trend did exist (Juel et al., 2004). Bickham et al. (2006) found that MCT4 protein content was not enhanced with 6 weeks of sprint-training in moderately endurance conditioned runners (in mixed muscle homogenates that contained both fast and slow twitch muscle fibers) even though MCT1 protein was increased. Perhaps Dubouchaud and coworkers (2000) are more accurate in their conclusion that MCT4 expression as the result of endurance training showed large interindividual variation before training and changed by variable amount due to training; thus, total muscle MCT4 contents did not change significantly due to training.

The effect of conditioning on MCT4 protein expression has not been documented in horses; however, Koho and associates (2006) speculated that interval type training, such as for Standardbred race horses that aims to increase anaerobic endurance and

gradually increases the accumulation of lactic acid, could trigger increases in MCT4 expression in equine muscle cells. Even though research in humans has failed to find a consistent correlation between increased MCT4 protein expression and high-intensity training (Pilegaard et al., 1999a; Juel et al., 2004; Thomas et al., 2005; Bickham et al., 2006), the higher arterial and venous [LA] in trained subjects did elicit an increase in MCT4 expression; exercise intensity was adequate to obtain a rise in [LA] (Pilegaard et al., 1999a). Juel et al. (2004) did report similar LA response in their research subjects; however, the authors did not find a statistically significant upregulation of MCT4 protein. Conditioning does not necessarily result in increases in MCT4 protein content in human athletic populations whereas protein expression of MCT1 appears to show more consistent responses to exercise.

Monocarboxylate transporter 1 cytosolic protein expression in the current study increased somewhat with conditioning; nonetheless, no statistically significant difference was found between the two treatment groups. However, MCT4 cytosolic protein content did not increase in as a result of conditioning. These findings are in agreement with Dubouchaud and colleagues (2000) who reported an approximately 90% increase in total muscle MCT1 protein expression in total muscle homogenates from human vastus lateralis muscle while MCT4 content did not change in response to conditioning.

The current study only evaluated transporter protein content in cytosolic fractions of whole muscle homogenates of the middle gluteal in horses. Immunohistochemical findings by light microscopy and TEM performed in a study by Kobayashi (2004), found that MCT1 and MCT4 are uniformly expressed on the plasma membrane of skeletal muscle fibers and that MCT4 is also expressed in vesicular structures adjacent to the

plasma membrane which would allow LA to be exchanged at the plasma membrane. Monocarboxylate transporter 1 is also found in inner mitochondrial membrane (Brooks et al., 1999). Western blot analysis by Dubouchaud et al. (2000) confirmed these findings in that MCT1 was located in the total muscle, mitochondrial, as well as sarcolemmal skeletal muscle fractions while MCT4 was absent in the mitochondria, but is suggested to be a constitutive sarcolemmal lactate transporter isoform due to its presence there. While MCT1 contents in the mitochondrial fractions increased after training, there was no change in MCT4 protein contents, but both MCT1 and MCT4 expression increased in the sarcolemmal fractions as a response to training (Dubouchaud et al., 2000).

Neither MCT1 nor MCT4 were found in the trans-Golgi networks (TGN) or in the intracytoplasmic tubulovesicular system (Kobayashi, 2004). The author suggests that MCT1 and MCT4 do not translocate from inside the cell to the cytoplasmic membrane under the influence of some sort of stimulus (Kobayashi, 2004) because while GLUT4 is located at those sites when muscle is inactive, it then translocates towards the plasma membrane upon stimulation by insulin or muscular contraction (Roy and Marette, 1996; Ploug et al., 1998). However, a stimulus of some sort must upregulate protein content of the monocarboxylate transporters as Coles et al. (2004) found that MCT1 and MCT4 were very rapidly up-regulated by a single exercise session. Protein content was measured by Western blot detection for 24 hours after a single 2 hour exercise session. Levels of MCT1 and MCT4 protein increased immediately after (2 hours post-exercise) and in varying amounts in three different muscle groups (soleus, red gastrocnemius, white gastrocnemius) for 24 hours post-exercise (Coles et al., 2004). Maximal protein content of MCT1 occurred 5-10 hours after exercise while MCT4 also peaked 5-10 hours

after exercise, but remained elevated to 24 hours post-exercise (Coles et al., 2004). Nonetheless, it was unclear as to which cellular fraction was used for Western blot analysis in the study by Coles and colleagues (2004).

AMP-activated protein kinase (AMPK) is known to translocate both GLUT4 and FAT/CD36 upon its phosphorylation and subsequent activation (Pandke et al., 2008); therefore it is plausible that AMPK could have the same result on MCT1 and MCT4. However, the AMPK activator, 5-aminoimidazole-4-carboxamide-1- β -D-ribose (AICAR) decreased MCT1 mRNA but did increase MCT4 mRNA expression in rat Sertoli cells (Galardo et al., 2007). There exists a positive relationship between MCT1 mRNA and MCT1 protein, but a lack of a relationship was found between MCT4 mRNA and MCT4 protein indicating that this protein is regulated by posttranscriptional mechanisms and MCT1 expression is regulated both by transcriptional and posttranscriptional mechanisms as suggested by Bonen et al. (2000).

Bonen et al. (2000) also reported that MCT4 was present in intracellular pools which could provide a reservoir of transporters that can be translocated to the plasma membrane upon some sort of stimulus such as contraction. This could be particularly important during times of high-intensity exercise when MCT4 could assist in the extrusion of LA from muscle cells. However, it is not presently known if intracellular MCT4 is able to be translocated to the plasma membrane as its concentration in the intracellular pools is only 25% of that that in the plasma membrane as compared to GLUT4 which has an intracellular concentration 5-8 times that of its plasma membrane concentration at rest (Roy and Marette, 1996; Bonen et al., 2000).

The subcellular fractions of MCT4 were 2.6 times greater than MCT1 in the T-tubules (TT) and 2 times greater than MCT1 in the triads (TR). Furthermore, MCT4 content was nearly 3 times greater than MCT1 in the sarcoplasmic reticulum (SR) fraction. In contrast, MCT1 is present in only very small amounts in the intracellular pool; little or no MCT1 was found in the intracellular membrane (IM) fraction (1.7%) although a substantial amount of MCT4 was present (24%) (Bonen et al., 2000). Although the presence of MCT1 is known in mitochondrial protein fractions (Brooks et al., 1999) the mitochondria is not an organelle in which transport proteins are cycled to the plasma membrane (Tonouchi et al., 2002). Furthermore, neither MCT1 nor MCT4 were found in the cytosolic compartment of sarcolemmal giant vesicles either at rest or upon electrical stimulation (Tonouchi et al., 2002); no assumptions were made as to whether the composition of this compartment is similar to the cytosol of the intact muscle.

As with MCT1, MCT4 protein expression does not appear to be influenced by diet type in horses and conditioning effects are highly variable. It remains to be determined if MCT4 protein expression can be enhanced by exercise. It has been suggested that MCT4 protein expression can be increased in equine skeletal muscle with exercise that increases lactic acid accumulation, but this conclusion is highly speculative at this point as results in human studies failed to find consistent correlation between MCT4 protein expression and high intensity exercise. Furthermore, the exact mechanism that contributes to the upregulation of these two proteins remains unclear at this point.

Table 14. Least squares means, standard errors (in parenthesis), and observed significance levels of effects on expression of transporter proteins in equine muscle by treatment and period, arbitrary units

Transporter	Treatment				P-value	
	Diet ¹		Post-exercise ²			
	Fat (n = 7) ³	Starch (n = 9)	Fat (n = 7)	Starch (n = 9)	Diet	Post - exercise
Fat/CD36	104.520 ^a (8.264)	78.138 ^b (7.729)	105.67 (14.981)	91.608 (14.318)	0.006	0.273
GLUT4	163.560 (9.958)	169.020 (8.782)	89.216 (7.514)	98.407 (6.627)	0.687	0.375
MCT1	108.210 (24.208)	86.775 (21.350)	142.420 (26.308)	135.740 (23.202)	0.517	0.852
MCT4	111.770 (25.088)	105.900 (23.086)	111.930 ⁴ (14.147)	106.690 ⁴ (13.563)	0.833	0.656

¹Time 1 (baseline/pre-treatment) to Time 2 (post-treatment/pre-exercise) comparison

²Time 2 (post-treatment/pre-exercise) to Time 3 (post-exercise) comparison

³Fat group has an n of 7 as one horse did not complete the exercise challenge

⁴Least squares means adjusted for the average MCT4 value of the pre-exercise (time 2) covariate (109.76)

^{a,b}Within a row and treatment, means that do not have a common superscript differ ($P < 0.05$)

CHAPTER V

CONCLUSIONS AND IMPLICATIONS

Previous research in the equine species has proven that supplemental fat can be fed safely and is effective for increasing the energy density of horse diets without the harmful metabolic effects of excessive starch intake. The premise of this study was to determine what adaptations take place within the myocyte in response to a high-fat diet that might better enable athletes such as horses to utilize dietary fat as metabolic currency.

The digestibility findings of this study were consistent with other published research in horses. Furthermore, feeding fat to yearlings during an aerobic conditioning program did not cause a negative impact on digestibility of other nutrients. In fact, supplementing the diets of yearlings with soybean oil enhanced digestibility of crude fat, crude protein, and calcium. Therefore, high fat diets consisting of supplemental soybean oil up to 10% of the total DM intake can be fed as safely and effectively to growing yearlings as a traditional high-starch ration provided that the total diet is balanced and nutrients are sufficiently available for growth and performance.

Skeletal growth patterns between the two treatment groups were not different and were consistent with other research in growing horses. Feeding supplemental fat to aerobically conditioned yearlings did not cause excessive weight gain despite an

increased consumption of DE as has been reported in trials using mature horses; therefore, no reduction in feed intake was necessary during the experiment to maintain appropriate body condition in the fat-fed horses. However, it was surprising that the horses on the Starch treatment were significantly heavier by the conclusion of the experiment than those on the Fat treatment despite the higher DE intake for those horses consuming supplemental fat. The reason(s) for this cannot be explained without further investigation. But meals with a high glycemic index such as those containing high amounts of dietary starch tend to promote glycogen synthesis due to a resulting increased insulin response which would increase membrane permeability to glucose (Snow et al., 1987). In addition, glycogen binds water while fat is stored anhydrous so the extra 5.6 kg of weight on horses consuming the starch diet could be wholly or partially be accounted for in water, but glycogen analysis or measurement of fat mass was not performed in the current study, so this conclusion is purely speculative at this point. The cause(s) for the increase in final weight for horses on the Starch treatment remain elusive without further examination.

Feeding supplemental fat in the form of soybean oil did not cause alterations in the blood metabolites, glucose, lactate, and triglycerides either at rest or after a 20 minute slow aerobic test at 4 m/s as compared to a traditional starch ration; ketogenic responses were variable. The exercise test was likely not challenging enough to the glucose homeostatic mechanism to cause alterations in plasma glucose between the two dietary groups. Likewise, lactate production was expected to be kept at a minimum during the slow aerobic test; the small amount of lactate that was produced could be immediately metabolized. Some of the published research on the effect of dietary fat on plasma TG

concentration indicates that dietary fat lowers plasma TG content. However, when Marchello and colleagues (2000) separated the various lipoprotein contents of the TG, they found that the VLDL fraction was greater in the fat-supplemented horses along with a sizeable decline in the LDL fraction. As VLDL lipoproteins are the primary lipoproteins responsible for transport of TG from the liver to adipose tissue and muscle (Murray et al., 2006), it could be speculated that more VLDL is available to use as fuel for the exercising muscle. Further examination would be required to determine if a) the fat supplemented horses had greater VLDL lipoprotein fractions, and b) the VLDL lipoprotein fraction was used as substrate for the working myocyte. The results for β -Hydroxybutyrate (BHB) indicated that a ketogenic diet would decrease the circulating levels of BHB at rest, but it was curious that BHB levels did not differ post-exercise. Could this mean that horses on the Fat treatment were able to utilize more BHB at rest? Decreased appearance, however, does not necessarily mean increased utilization so the consequences of this finding require further investigation. It was disappointing that the results of the acetoacetate analysis were unusable as the results may have shed some light on ketone production and availability in horses with respect to diet. It remains to be elucidated if an increase in exercise intensity and/or duration could have resulted in differences in post-exercise blood metabolites between the fat supplemented horses and controls in this particular study.

The endocrine response at rest and post-exercise did not differ by treatment for insulin or adiponectin but differences were evident for some horses in the plasma concentration of leptin at rest. Nor did the exercise intensity or duration performed cause significant differences in the heart rate response to exercise by dietary treatment. The

results of the leptin assay indicate that horses on the Fat treatment that had baseline values of 1.40 ng/mL HE or larger (n=5) had a larger mean response for leptin levels due to diet as compared to their Starch counterparts, but overall it can be said that there was no difference between treatments. Previous research in horses indicates that leptin levels are positively correlated with fat mass. Therefore, it can be ascertained that those horses with higher mean leptin responses exhibited a greater percentage of fat mass than those on the Starch treatment. This assumption is speculative at this point because fat mass was not measured in the current study. The hormone adiponectin, also related to fat mass although inversely so, did not differ by treatment or post-exercise, but not all data points were usable and when the baseline value was missing, the covariate analysis was impaired. Further examination including measurement of fat mass is required to determine unequivocally if feeding supplemental fat in the form of soybean oil as 10% of the total dry matter intake to aerobically conditioned yearlings could cause alterations in the plasma hormones leptin and adiponectin both at rest and post-exercise as compared to yearlings consuming a traditional high-starch ration. Exercise intensity and/ or duration, would also likely need to be increased to cause potential alterations in heart rate and insulin responses post-exercise due to dietary treatment.

The quantity of protein transporters GLUT4, MCT1 and MCT4 in the cytosol of the myocyte did not differ by treatment at rest or post-exercise. The novel finding of the current research is the increased quantity of the fatty acid transporter, FAT/CD36 in the skeletal muscle of horses on the Fat treatment. The increased amount of FAT/CD36 available in the cytosol of the myocyte could be of benefit for the clearance of LCFA during aerobic work. However, further analysis would need to take place which could

include separation of the various fractions of the muscle cell into cytosolic, sarcolemmal and mitochondrial fractions. The lack of a difference in post-exercise measures is likely due to the fact that sarcolemmal fractions were not assayed, so it is unknown what quantity of transporters could have been upregulated to the sarcolemmal upon stimulation by insulin or contraction as in the case for GLUT4 and FAT/CD36. To our knowledge, no published research exists which examines the effect of diet on the aforementioned substrate transporters in the muscle. The practices and conclusions of this study should stimulate further directive research in this area and potentially help define ergogenic benefits of fat supplementation with respect to endurance exercise in horses.

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VITA

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Scope and Method of Study: It is our hypothesis that feeding supplemental dietary fat as compared to a traditional high-starch ration will promote muscular adaptations that would enable greater fat utilization during exercise. Seventeen Quarter Horse yearlings with no previous conditioning were utilized in a randomized complete block design and were assigned one of two treatment diets FAT (n=8) using 10% soybean oil as the added fat, or STARCH (n=9). Horses were fed treatment diets while being aerobically conditioned for a period of 8 weeks. Body weights and skeletal measurements were taken weekly and a total fecal collection was performed in two periods evaluate impact of fat supplementation on growth patterns and nutrient digestibility. Blood parameters and muscle tissue were evaluated both at baseline and post-treatment to determine if the capacity to transport fatty acid substrates from the blood stream to the myocyte can be increased. Data collected during the first week of the study were used as baseline measures and diets were compared using analysis of covariance (ANCOVA) methods. A repeated measures analysis modeling correlation structure was done for the digestibility variables. The SAS/MIXED procedure was used for these analyses. All tests were performed at the $P < 0.05$ level of significance.

Findings and Conclusions: There was improved digestibility of crude fat, crude protein and Ca in the FAT horses. STARCH horses had significantly heavier body weights by the end of the treatment period, but no difference between dietary treatments existed for skeletal measurements. STARCH horses had higher plasma betahydroxybutyrate concentrations post-treatment. FAT horses had higher fatty acid translocase (FAT/CD36) cytosolic protein expression in the myocyte post-treatment. These data suggest that fat supplementation at the inclusion rate of 10% soybean oil (DM basis) in diets of growing horses subjected to aerobic conditioning could potentially enhance the capacity for transport of fatty acid substrates as defined by an increased expression of the FAT/CD36 protein in the myocyte. Feeding a high-fat diet during conditioning could improve the capacity of horses to utilize fat as a substrate. The practices and conclusions of this study should stimulate further research to define ergogenic benefits of fat supplementation in diets fed to endurance horses.

ADVISER'S APPROVAL: Dr. Steven R. Cooper
