RUMINANT SUPPLEMENTATION OF GLUCONEOGENIC SUBSTRATES VIA DRINKING WATER

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ABSTRACT

Dark cutting or high pH meat is found in all meat species, inducing significant losses to the meat industry. Dark cutting meat is a direct consequence of low muscle glycogen at slaughter, which affects the normal acidification of meat during rigor development, hence, the ultimate pH remains high. The ultimate pH of the meat has a great influence on certain meat quality parameters, such as color, water holding capacity, and tenderness. An animal exposed to stress previous to slaughter may significantly depletes its glycogen reserves. This situation leads to a high ultimate pH if the animal is slaughtered before it has sufficient time to replenish their muscle glycogen stores. Due to stress during pre-slaughter handling is an inevitable process, a strategy to mitigate its effect may be increase muscle glycogen levels prior to slaughter.

Supplementation of ruminants prior to slaughter with glycerol or fructose as a source of gluconeogenic substrates may improve muscle glycogen concentrations at slaughter and meat quality. However, dietary carbohydrates are extensively fermented in the rumen and less than 10% of the glucose requirement is absorbed from the digestive tract, thus gluconeogenesis must provide around 90% of the total glucose needs in ruminants. It has been shown 60-80% of drinking water bypasses the rumen. Therefore, gluconeogenic substrates supplemented via drinking water may escape microbial fermentation in the rumen and be absorbed in the small intestine to be available for utilization by the tissues.

In experiment 1, described in chapter two, Angus-cross steers (n=36) received 4.3% crude glycerin or high fructose corn syrup via drinking water in addition to a high concentrate diet. The objective of this study was to supply substrates for gluconeogenesis and *de novo* fatty acid synthesis via drinking water to increase marbling and glycogen concentration in the muscle. Overall, crude glycerin or high fructose corn syrup supplementation via drinking water did not alter carcass or meat quality variables but did alter the size and distribution of intramuscular adipocytes. These results indicate that a longer supplementation time or a higher substrate level may be needed in order to obtain differences in meat quality.

In experiment 2, described in chapter three, Southdown wether lambs (n=18) were used to investigate the effect of glycerol or fructose at 12% via drinking during 28-d grazing period and 2-d fasting previous to slaughter on animal performance, postmortem glycolysis and pH decline, proximate and fatty acid composition of tissues, and changes in gene expression of enzymes involved in lipid and glucose metabolism in liver. Overall, glycerol supplementation increased ADG during the grazing period, reduced BW shrink during fasting, and increased HCW. Glycerol supplementation favored muscle glycogen at early postmortem times and glycogen content in the liver, decreased lipid mobilization during fastening and upregulated mRNA expression of lipogenic and glucose transport genes in liver.

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CHAPTER ONE

LITERATURE REVIEW

INTRODUCTION

Meat color is the first visual selection criterion that consumers use as an indicator of meat quality. It has great influence on the final decision of purchase, since consumers use discoloration as an indicator of spoilage and shelf life (Mancini, 2009). Dark cutting or dark, firm, and dry (DFD) meat is a direct consequence of low muscle glycogen at slaughter, which affects the normal acidification of meat during rigor development, hence, the ultimate pH remains high (Tarrant, 1989). The ultimate pH affects the color of the meat; the dark purplish-red color characteristic of dark cutting meats results unattractive for the consumer, affecting negatively their acceptability (Viljoen et al., 2002). Furthermore, shelf life and palatability are often impaired in dark cutting meats (Viljoen et al., 2002; Faucitano et al., 2010). Dark cutting or high pH meat is found in all meat species, inducing significant losses to the meat industry (Adzitey and Nurul, 2011).

Marbling is an important meat quality factor which affects flavor, juiciness and tenderness perception by consumers (Hausman and Poulos, 2009). In the beef industry, the US Department of Agriculture uses marbling as a major factor in establishing beef carcass quality grade, which is an indicator of eating quality. Additionally, yield grade is used to estimate cutability, and it is highly influenced by the amount of fat thickness at the 12th rib in the carcass (Ramsey et al., 1962). The increase in the degree of marbling

can result in a premium price of the carcass. It has been shown that the probability of consumers purchasing beef steaks and the price they are willing to pay increase as marbling score increases (Platter et al., 2005). Typically the increase in marbling can be reached with a greater time-on-feed, however, the increase in fat thickness in cattle beyond a specific point will not ensure increased marbling or quality grade (Garcia et al., 2008). Excess subcutaneous fat decreases the carcass value, represents a higher feeding cost for the producers and increases waste for the industry (Du et al., 2013).

MUSCLE AND LIVER GLYCOGEN METABOLISM

Glycogen is the major storage carbohydrate in animals. It is a branched homo-polysaccharide formed by glucose monomers linked by α1,4 glucosidic bonds with branching by α1,6 glucosidic bonds (Bender and Mayes, 2012a). In both muscle and liver, glycogen forms spherical granules which contain up to 60,000 glucose residues (Bender and Mayes, 2012a). Glycogen constitutes 1 to 2% of the total muscle mass antemortem and up to 10% of the liver mass (Keeton, et al. 2009). In the muscle, glycogen is used as a source of energy for muscle contraction and relaxation, whereas in the liver glycogen is used for maintaining blood glucose concentration during the fasted state in monogastrics and during the fed and fasted states in ruminants.

During glycogenesis, glucose is phosphorylated to glucose 6-phosphate by hexokinase in muscle and glucokinase in liver. Glucose 6-phosphate is isomerized to glucose 1-phosphate by phosphoglucomutase. In the initial step in glycogen synthesis, the

protein glycogenin form a glycogen primer with glucose residues, necessary for the activity of glycogen synthase (Poso and Puolanne, 2005). Glycogen synthase, encoded by the gene GYS2, catalyzes the elongation of glycogen by α1,4-glycosyl bonds. Branching enzyme, encoded by the gene GBE1, transfers a part of the α1,4 chain to a neighboring chain forming an α1,6 linkage, establishing a branching point. The branches grow by additions of α1,4 glucoses and further branching (Bender and Mayes, 2012b).

The enzyme glycogen phosphorylase catalyzes the rate-limiting step in glycogenolysis, the breakdown of glycogen to glucose 1-phosphate which is utilized in glycolysis for ATP for muscle contraction. The catalytic subunit of the enzyme is bound to glycogen whereas the other subunit regulates the function of the enzyme. Glycogen phosphorylase cleaves glucose residues from the non-reducing end of the unbranched chains until the fourth glucosyl unit from the branching point (Poso and Puolanne, 2005). There are different isoenzymes of glycogen phosphorylase in liver, muscle and brain, encoded by the genes PYGL, PYGM and PYGB, respectively. The debranching enzyme, encoded by the gene GDE, has two distinct catalytic sites. When phosphorylase has cleaved the glucosyl units to the level of 4 units from the branching point, the transferase site transfers a trisaccharide unit from the α1,6 branching point, whereas the 1,6-glucosidase site cleaves the remaining α1,6-glucosyl unit as free glucose (Poso and Puolanne, 2005). Free glucose is further phosphorylated (Bender and Mayes, 2012b). In liver, glucose 6-phosphatase, encoded by the gene G6PC, catalyzes the hydrolysis of glucose 6-phosphate to glucose, which can be exported to increase blood glucose

concentration. Glucose 6-phosphatase is only present in liver; in muscle glucose 6-phosphate is used for glycolysis to provide ATP for muscle contraction.

The activity of phosphorylase on glycogen molecules with low number of non-reducing ends is reduced due to spatial reasons, therefore, the glycogen molecule may not be fully utilized (Poso and Puolanne, 2005). Immonen and Puolanne (2000) evaluated the relationship between ultimate pH and residual glycogen concentration in cattle. Residual glycogen varied from 10 to 80 mmol/kg at an ultimate pH range of 5.4-5.75. These values correspond to glycogen concentrations of about 55 to 125 mmol/kg at slaughter. At pH higher than 5.75, there was always 10-20 mmol/kg glycogen left in muscles, indicating that a minimum residual glycogen concentration may exist.

The enzymes glycogen phosphorylase and glycogen synthase are regulated in opposite directions by reversible phosphorylation and dephosphorylation. Phosphorylation is catalyzed by the enzyme phosphorylase kinase, whereas dephosphorylation is catalyzed by phosphoprotein phosphatase. Phosphorylation of glycogen phosphorylase increases its activity, whereas phosphorylation of glycogen synthase reduces its activity. Phosphorylation is increased in response to cyclic AMP, which is formed from ATP by adenylyl cyclase in response to hormones such as epinephrine, norepinephrine, and glucagon. Cyclic AMP activates the enzyme protein kinase, which catalyzes the phosphorylation of inactive phosphorylase kinase to active phosphorylase kinase, which in turn activates the enzyme glycogen phosphorylase by phosphorylation.

In liver, cyclic AMP is formed in response to glucagon, which is secreted by the α-cells of the pancreatic islets, to increase blood glucose. Glucagon does not have an effect in muscle phosphorylase. In muscle, cyclic AMP is formed in response to epinephrine, which binds to β-adrenergic receptors on the muscle fiber membrane. Epinephrine is liberated from the adrenal medulla in response to fear or fright to increase glycogenolysis for rapid muscle activity. Under prolonged stress, this mechanism induces glycogen depletion which may be detrimental for meat quality if it occurs before slaughter.

BIOCHEMICAL CHANGES POSTMORTEM AND DARK CUTTING CONDITION

Muscle glycogen reserves are used as an energy source for muscle contraction and relaxation. Lactic acid is a byproduct of glycogen utilization by the muscle in anaerobic conditions and, in the living animal, it can be oxidized in heart, liver and aerobic muscles or converted back to glucose by gluconeogenesis in the liver (Poso and Puolanne, 2005). After death, the blood circulation is interrupted, oxygen supply to the muscle ceases and the removal of metabolites also stops. The muscle metabolism is modified towards anaerobic glycolysis, and glycogen is consumed to produce energy, producing lactate that accumulates in the muscle. During the first hours postmortem, calcium is released to the cytosol inducing muscle contraction. This leads to exhaustion of ATP reserves limiting the dissociation of actin-miosin complex, resulting in the development of rigor mortis. The accumulation of lactic acid is responsible for the pH decline postmortem to an

ultimate pH of 5.4 to 5.8 during normal rigor mortis development (Poso and Puolanne, 2005).

One of the main factors determining the rate of postmortem metabolism is muscle fiber type composition (Ryu and Kim, 2005). In mammals, there are 4 main types of muscle fibers which are classified by their contractile and metabolic properties: slow twitch type I (red) fibers characterized by an oxidative metabolism, fast twitch type IIA and type IIX fibers with an intermediate metabolism oxido-glycolitic, and fast twitch type IIB (white) fibers with a glycolytic metabolism (Lefaucheur and Gerrard, 2000). The proportion of each type of fiber in a muscle depends on the muscle type, location, and function (Lefaucheur and Gerrard, 2000). Muscles involved in posture are more oxidative than those involved in movements (Klont et al., 1998). For example, *Supraspinatus* is essentially an oxidative slow twitch muscle, *Longissimus* is a fast twitch red muscle, having both high oxidative and high glycolytic activity, and *Semitendinosus* is a glycolytic fast twitch muscle (Briand et al., 1981; Vestergaard et al., 2000). In general, glycolysis and rigor mortis development are faster in white glycolytic than in red oxidative muscles (Klont et al., 1998; Dransfield and Sosnicki, 1999). Ultimate pH and pH at 45 min postmortem has been shown to be inversely related to the number of type IIB (glycolytic) fibers, and positively related to the number of type I (oxidative) and type IIA (oxido-glycolitic) fibers (Ryu and Kim, 2005).

The ultimate pH is directly proportional to the accumulation of lactic acid, which depends on the concentration of glycogen previous to slaughter. If muscle glycogen reserves at slaughter are normal and the animal is not exposed to stress antemortem, the

pH decrease normally until anaerobic glycolysis ceases when the pH has reached 5.6 to 5.3, even in the presence of large amounts of residual glycogen (Immonen and Poulanne, 2000). A normal pH decline (Figure 1.1) is characterized by a gradual decrease from approximately 7.4 in living muscle to 5.6-5.7 within 6 to 8 h postmortem, reaching an ultimate pH at approximately 24 h postmortem of 5.3-5.7 (Aberle et al. 2012). However, if the glycogen content before slaughter is low, as a result of an insufficient dietary supply or due to long-term stress that causes the depletion of glycogen prior to slaughter, lactic acid accumulation is limited (Warriss, 2000). In this case, the pH drops only slightly during the first hour postmortem (Aberle et al., 2012) and the ultimate pH remains high (Lahucky et al., 1998) (Figure 1.1). A high ultimate pH (above 5.8) increases the incidence of DFD meat (Warriss, 2000). Lowe et al. (2004) reported that low concentrations of glycogen in the muscle measured 45 min postmortem (below 25 μmol/g of fresh tissue) resulted in high ultimate pH of the meat (above 5.9). Pale, soft, and exudative (PSE) meat is characterized by low pH, a pale and exudative appearance, and a soft texture (Shen et al., 2009). This condition, results from a rapid glycolysis postmortem and a pH drop to around 5.4-5.5 during the first hour postmortem, whereas the ultimate pH remains in the range of 5.3 to 5.6 (Aberle et al., 2012) (Figure 1.1). It is generated when animals are expose to acute stress just before slaughtering (Adzitey and Nurul, 2011).

Ultimate pH has a great influence on certain meat quality parameters, such as color, water holding capacity (WHC), and tenderness (Gregory, 2003). Water holding capacity is the ability of meat to retain water during cutting, heating, and pressing. A higher WHC

results in a dry surface of the lean, characteristic of DFD meats. The capacity of muscle proteins to bind water is influenced by the meat pH. At high pH, the proteins are above their isoelectric point, in a highly ionized state, and bind water tightly increasing the WHC of meat (Hamm, 1974; Huff-Lonergan and Lonergan, 2005). In PSE meat, the rapid glycolysis postmortem generates significant amounts of heat, which increases the carcass temperature (Shen et al., 2009). The combination of a low pH in muscle and the rise in temperature cause denaturation of muscle proteins, leading to reduction of their capacity to bind water and generally a loss of color intensity (Aberle et al., 2012).

There are several procedures available for measuring the WHC of meat and meat products in raw and cooked meat; the more common methods described in the literature include drip loss, filter paper press method, centrifugation, and cooking loss (Trout, 1988; Honikel 1998). These methods measure the amount of water expelled; a higher loss indicates lower WHC. Several studies have shown a positive correlation between meat pH and WHC in different species, such as pigs (McCaw et al., 1997; Joo et al., 1999; Leheska et al., 2003), sheep (Apple et al., 1995; Miranda-de la Lama et al., 2009), broiler chickens (Dadgar et al., 2012; Sheard et al., 2012) and beef (Lahucky et al., 1998).

Dark cutting meat exhibits a dark purplish-red lean color, which in a retail display is a major cause of rejection by the consumers, since consumers associate the dark color to meat from several undesirable traits, like meat from an old animal, spoilage, off-flavors, toughness, and poor shelf life (Miller, 2007). Several factors determine the dark color of DFD meats. As a consequence of the higher power to bind water, the muscle fibers are tightly packed resulting in a closed structure with small extracellular space.

This condition reduces the diffusion of oxygen into the muscle from the surface. Additionally, the consumption of oxygen by mitochondria is higher due to the high pH (Warriss, 2000). The consequent lower oxygen availability associated with high ultimate pH in meat determines a lower association between myoglobin and oxygen, decreasing the proportion of oxymyoglobin in the muscle (AMSA, 2012). Dark cutting meat shows only a very thin surface layer of bright-red oxymyoglobin that allows the purple color of the underlying reduced deoxymyoglobin to show through, resulting in a darker color (Warriss, 2000). The darker color also results from a lower light reflectance or light scattering in the surface of the closed structure that characterizes DFD meat compared to a more open structure in meats of lower pH (Abril et al., 2001). Several authors reported a negative relationship between ultimate pH and meat color; higher ultimate pH values were associated with lower lightness and redness, showing a darker meat color (Guignot et al., 1994; Wulf et al., 1997; Abril et al., 2001).

Additionally, the closed structure that results from the tightly packed muscle fibers make DFD meats firm. However, the relationship between meat ultimate pH and tenderness is controversial. Some authors found a curvilinear relationship between pH and shear force values, with maximum toughness (higher shear force values) at ultimate pH values between 6.0 and 6.3 (Watanabe et al., 1996, Jelenikova et al., 2008). Others have reported a linear relationship between ultimate pH and tenderness, with more tender meat (lower shear force values) at higher ultimate pH (Guignot et al., 1994; Silva et al., 1999). Silva et al. (1999) suggested that a higher proteolytic activity is responsible for the degradation of myofibrillar proteins at higher pH, while in normal pH meat, proteolysis

would be slower. In accordance to this, Beltran et al. (1997) showed higher tenderness and m-calpain activity in meat from young bulls of higher ultimate pH.

The high pH that characterizes DFD meat increases the spoilage potential, because a high pH is favorable to microbial growth (Newton and Gill, 1981; Faucitano et al., 2010). Furthermore, DFD meat is characterized by very low levels of glucose and glycolytic intermediates. Therefore, the growth of lactic acid producing bacteria is restricted and this encourages the growth of bacteria that metabolize amino acids and proteins, which produce undesirable spoilage odors (Newton and Gill, 1981; Wariss, 2000). The increased spoilage and undesirable flavor due to microbial growth shortens meat shelf life (Newton and Gill, 1980).

Dark cutting condition causes significant losses to the meat industry. It has been estimated that the dark cutting condition costs the U.S. beef industry between $ 132 and $172 million annually (Wulf et al., 2002; Apple et al., 2005). According the National Beef Quality Audit, the incidence of dark cutting was 2.7% in 1995 (Wulf et al., 2002), 1.9% in 2005 (Garcia et al., 2008), and 0.85% in 2011 (Gray et al., 2012). However, it is possible to have a greater incidence of dark cutting meat in a group of cattle more susceptible to stress before slaughter.

ANTE-MORTEM FACTORS AFFECTING GLYCOGEN CONTENT POSTMORTEM

As previously mentioned, muscle glycogen is the main metabolic substrate responsible for postmortem lactic acid accumulation and normal ultimate pH of the meat.

Glycogen concentration before slaughter varies depending on the muscle (Hambrecht et al., 2005), the nutritional status (Geay et al., 2001), and the level of pre-slaughter stress (Immonen and Puolanne, 2000). Stress exposure prior to slaughter may have detrimental effects on meat quality (Tarrant and Grandin, 2000; Maria, 2008). Ante-mortem stress includes the conditions and practices that apply during the period when the animal is moved on-farm to entry into the knocking box at the slaughterhouse. Therefore, animals are exposed to several stimuli including handling and increased human contact, transportation, fear, unfamiliar environment, food and water deprivation, lairage time, changes in social structure, and changes in climatic conditions (Ferguson and Warner, 2008). An animal exposed to stress previous to slaughter may significantly deplete its glycogen reserves, leading to a high ultimate pH. Any situation which provokes a substantial depletion of muscle glycogen reserves will result in meat with a high ultimate pH if the animal is slaughtered before it has sufficient time to replenish their muscle glycogen stores (Silva et al., 1999). Replenishment of muscle glycogen reserves may take a few days or as long as two weeks post-stress. McVeigh and Tarrant (1982) studied the rate of recovery after glycogen depletion by adrenalin injection. The authors reported higher rates of repletion for heifers fed barley or hay (7.6 and 6.1 µmol/g per day, respectively) and significantly lower values for fasted heifers (1.5 µmol/g per day). It has been shown that there is a positive relationship between metabolizable energy intake and rate of muscle glycogen repletion following exercise stress in cattle, whereas sheep respond at lower levels of metabolizable energy intake (Gardner et al., 2001). In general, high energy diets are more effective than forage based diets in repleting muscle glycogen.

Immonen et al. (2000a) reported greater muscle glycogen repletion rates with a high concentrate diet compared to alfalfa haylage in steers and bulls following adrenaline depletion. Ruminants' repletion rates are substantially lower than that reported for monogastrics. Terjunk et al. (1974) reported glycogen repletion rates between 14.7 and 41.6 μmol/g per hour in different muscles of rats after strenuous exercise. In humans, Peters Futre et al. (1987) reported a glycogen repletion rate of 16.8 μmol/g per hour after intense short-term exercise. According to Tarrant (1989), the lower repletion rates found in ruminants are due to a lower glucose availability compared with non-ruminants.

The exhaustion of animals after social regrouping due to high level of physical activity (aggressive behavior, mounting activity, emotional excitement, establishment of a new social hierarchy) may be a cause of dark cutting meat, especially in bulls or animals of high temperament. McVeigh and Tarrant (1983) induced stress in young bulls by mixing unfamiliar animals during a 5-h period. The resting muscle glycogen content decreased from 87 μmol/g of wet tissue to 32 μmol/g of wet tissue at the end of the stress period. Muscle glycogen repletion rates during a 3-d recovery period after stress were 14.8 μmol/g per day. These results show clearly the differences in depletion and repletion rates; in this example more than 3 d were necessary to recover the glycogen depleted in 5 h of stress.

The influence of stress due to transportation on meat quality will vary depending on the conditions during the journey and the duration of transportation. Long transport time was associated with high muscle ultimate pH and darker meat (Gallo et al., 2003). The increase in incidence of dark cutting after long distance transport could be reversed by

resting and feeding the animals for 2 days or longer before slaughter (Warriss et al., 1984; Tarrant and Grandin, 2000). In some cases, glycogen reserves can be restored at lairage even if animals are not fed (Warriss et al., 1984; Mounier et al., 2006), but it will depend of the stress level during lairage. Others (Gallo et al., 2003) observed an increase in the proportion of dark cutting carcasses with increased lairage periods without food. Generally, if the transport time is short, driving conditions are good and the space allowances are in the range of recommended values for each specie and category, transport should not be a major factor affecting meat quality. Grigor et al. (2004) did not find any effect on ultimate pH and meat quality of calves transported for 3 h compared to non-transported claves. However, the transport and handling were stressful to the calves, resulting in less resting behavior, greater disturbance and increased heart rate, plasma cortisol concentration and plasma creatine kinase activity. Similarly, increasing the journey time from 3 to 8 h did not impair ultimate pH, color, tenderness, WHC or sensory characteristics of beef (Alende et al., 2014).

Adverse seasonal conditions during transportation and lairage may increase the stress conditions of the animals and affect negatively carcass and meat quality characteristics. Miranda-de la Lama et al. (2009) reported that meat from lambs slaughtered in winter in Spain presented some characteristics of dark cutting meat, with darker color, higher ultimate pH, less tenderness and more water holding capacity than lambs slaughtered in summer. In US the 2011 National Beef Quality Audit (Gray et al., 2012) reported that 62 % of the dark cutters occurred during the months of September through January, most likely reflecting environmental stress during that period.

According to Tarrant (1989), glycogen depletion in living muscle may be triggered by either increased catecholamine (epinephrine) levels or muscle contraction, or by both mechanisms acting in concert. Additionally, a slow depletion of muscle glycogen occurs during starvation. Epinephrine modifies the energetic metabolism inducing muscle glycogenolysis through the activation of the enzyme phosphorylase (Wariss, 2000). Plasma concentrations of epinephrine were elevated in sheep in response to a 6-h period of restraint and isolation stress immediately previous to slaughter (Apple et al., 1995). As a result, glycogen reserves decreased before slaughter, and meat glycogen and lactate concentrations were decreased, affecting normal postmortem pH decline. The ultimate pH was higher in meat from stressed sheep, leading to the formation of the dark cutting condition. Similar results were reported in cattle subjected to restraint and isolation previous to slaughter (Apple et al., 2005).

In cattle and sheep that were not exposed to stress, normal muscle glycogen levels typically ranges from 65 to 120 μmol/g (Apaolaza et al., 2015). Resting muscle glycogen content is influenced by energy intake; in general, animals fed high energy diets exhibit higher glycogen content than animals fed low energy diets. McVeigh and Tarrant (1982) reported higher values of muscle glycogen for heifers fed barley compared to heifers fed hay. Vestergaard et al. (2000) reported that bull calves fed forage-based diets had less glycogen, higher muscle pH, and darker muscle color than bulls fed ad libitum concentrates. Similarly, lambs finished on grain-based feedlot rations had higher muscle glycogen concentrations than lambs finished on pasture (Jacob et al., 2005). These results may be due to a higher ruminal production of propionate with high energy diets (Bauman

et al., 1971). In ruminants, propionate is a major substrate for gluconeogenesis in the liver (Bender and Mayes, 2012b).

Due to stress during pre-slaughter handling is an inevitable process, a strategy to mitigate it may be increasing muscle glycogen levels prior to slaughter. Immonen et al. (2000b) studied the effect of a high-energy diet provided to bulls for 2 weeks prior to transportation to slaughter. The loss of glycogen during transportation was lower, glycogen content at the time of slaughter and residual glycogen were higher, and ultimate pH was lower with a high energy diet than low energy diet; the high energy diet seemed to protect cattle from potentially glycogen-depleting stressors such as high temperatures and transportation.

INTRAMUSCULAR FAT AND MARBLING

The term marbling is sometimes used as a synonymous of intramuscular fat, due to the fact that when intramuscular fat is abundant, it gives a marbled appearance to the lean (Warriss, 2000). However, intramuscular fat is a chemical entity which refers to the amount of fat that can be measured between the muscular fibers, whereas marbling is a visual indicator which aims to estimate the amount of intramuscular fat (Ngapo et al., 2013). Marbling has been associated with several factors affecting the overall evaluation of beef by consumers; a positive relationship between marbling and palatability attributes such as tenderness, juiciness, flavor and overall palatability has been reported in the literature (Wheeler et al., 1994; Jeremiah, 1996; Platter et al., 2003; Thompson, 2004).

Wood (1995) has suggested that improved sensorial tenderness might be due to the fact that fat is softer than the myofibrillar elements, therefore increasing the fragmentation of beef during chewing, allowing fiber bundles to separate from one another more easily. On the other hand, fat promotes the flow of saliva in the mouth, improving the juiciness perception, whereas flavor improvement might derive from reactions of the fat during cooking (Wood, 1995; Warriss, 2000).

Intramuscular fat is a late developing tissue in which hyperplasia plays a very important role (Cianzio et al., 1985; May et al., 1994; Yang et al., 2006). The development of intramuscular fat is correlated with other fat sites (i.e., visceral, intermuscular and subcutaneous) (Gerrard and Grant, 2003). Therefore, the desirable accumulation of intramuscular fat is generally associated with an increase in other fat depots which are not desirable and which represent an increase in the production costs (Du et al., 2013). Monogastrics and ruminants differ in the metabolic energy sources for fatty acid synthesis. Glucose is the main source in monogastrics animals, whereas acetate produced during ruminal fermentation is considered to be the main precursor for ruminant lipogenesis (Roh et al., 2006). However, Smith and Crouse (1984) demonstrated that the substrate specificity can vary with depot site; glucose rather than acetate seems to be the primary substrate for fatty acid synthesis in intramuscular adipose tissue of beef cattle. The greater importance of glucose as a lipogenic precursor in the intramuscular fat depot indicates a potential to manipulate fat deposition in other depots without adversely affecting marbling scores and palatability.

In experiments with primary adipocytes cultures from humans (Robubi et al., 2014), supplementation with fructose has been shown to increase the expression of genes involved in lipogenesis and hexose uptake and hasten the development of lipid vesicles compared to glucose. Studies *in vivo* have shown that high-fructose diets stimulate hepatic *de novo* lipogenesis, increases plasma triglycerides, total body lipid content, and insulin resistance in rats (Crescenzo et al., 2013, 2014) and humans (Stanhope et al., 2009). Fructose is metabolized mainly in the liver; hepatocytes convert fructose to fructose-1-phosphate by fructokinase (Mayes, 1993). However, Faeh et al. (2005) reported that a high-fructose diet induced adipose tissue insulin resistance; the authors speculated that a fraction of the dietary fructose may reach systemic circulation and act directly on adipose tissue.

GLUCONEOGENESIS AND SUPPLEMENTATION OF GLUCONEOGENIC SUBSTRATES

Gluconeogenesis is the process of synthesizing glucose from noncarbohydrate precursors. Gluconeogenesis is not the reversal of glycolysis; key enzymes in opposing metabolic pathways are regulated in order to achieve the net flux in the appropriated direction (Nordlie et al., 1999). Glycolysis is catalyzed by 3 key enzymes: hexokinase, which catalyzes the phosphorylation of glucose to glucose 6-phosphate, phosphofructokinase which phosphorylates fructose 6-phosphate to fructose 1,6-biphosphate, and pyruvate kinase, which converts phosphoenolpyruvate to pyruvate. The

reversal of the reaction catalyzed by pyruvate kinase involves two reactions catalyzed by mitochondrial pyruvate carboxylase and cytosolic phosphoenolpyruvate carboxykinase. Pyruvate carboxylase converts pyruvate to oxaloacetate, which is reduce to malate and is exported from the mitochondria to the cytosol and there oxidized back to oxaloacetate. The enzyme phosphoenolpyruvate carboxykinase catalyzes the decarboxylation and phosphorylation of oxaloacetate to phosphoenolpyruvate. The conversion of fructose 1,6-biphosphate to fructose 6-phosphate is catalyzed by fructose 1,6-biphosphatase. The conversion of glucose 6-phosphate to glucose is catalyzed by glucose 6-phosphatase. This enzyme is present in liver and kidney but absent from muscle and adipose tissue, which, therefore, cannot export glucose to the bloodstream. The relationships between gluconeogenesis and glycolysis are shown in Figure 1.2.

In ruminants, carbohydrates are extensively fermented in the rumen and less than 10% of the glucose requirement is absorbed from the digestive tract, thus gluconeogenesis must provide around 90% of the total glucose needs in ruminants (Young, 1977). Carbohydrates are fermented to volatile fatty acids in the rumen, mainly acetate, propionate and butyrate, but only propionate is a gluconeogenic substrate (Fahey and Berger, 1988). Other substrates for gluconeogenesis are glycerol, lactate, and glucogenic amino acids (Nafikov and Beitz, 2007).

Gluconeogenic substrates enter the glucose metabolism pathway in different stages (Figure 1.2). Propionate enters gluconeogenesis pathway via the citric acid cycle. Lactate enters the pathway previous conversion to pyruvate, whereas glucogenic amino acids are converted to either pyruvate or intermediates of the citric acid cycle by transamination or

deamination. Glycerol is phosphorylated by glycerol kinase and enters gluconeogenesis as glycerol 3-phosphate. In the case of fructose, it is rapidly converted to fructose-1-phosphate in the liver by fructokinase. Then it is split by aldolase into dihydroxyacetone phosphate and glyceraldehyde, which is further phosphorylated to glyceraldehyde 3-phosphate by triokinase. Thus, fructose enters glucose metabolism at the triose phosphate stage, without passing the main rate-controlling step in glycolysis catalyzed by phosphofructokinase (Mayes, 1993).

Drinking water has been used as a mean of supplementation of gluconeogenic substrates in dairy cows, beef and sheep. In dairy cows, 2% glucose (Osborne et al., 2002) or 2% glycerin (Osborne et al., 2009) were delivered via drinking water to periparturient cows, in an attempt to deliver energy substrates postruminally during the transition period when the feed intake is reduced. Bulls subjected to stress of mixing, handling and transportation previous to slaughter were supplemented with 5% glucose or electrolytes via drinking water during the lairage period in the slaughterhouse (Schaefer et al., 1990). The authors observed an increase in carcass weight of 3-4% with glucose and electrolyte solutions, due to improved electrolyte balance. In sheep, Gardner et al. (2001) reported an increase in plasma glucose concentrations with water containing 3.5% glycerol and 1.5% propylene glycol over 24 h period. The authors reported a greater hyperglycemic effect in animals fed roughage compared to sheep fed high energy diets, which may indicate a greater effect of these supplements in pasture fed than in feedlot animals. When glycerol/propylene glycol solution was administered for 48 h post

exercise in the absence of food, muscle glycogen concentration was increased (Gardner et al., 2001).

Supplementation of ruminants with gluconeogenic substrates may be an interesting strategy to increase muscle glycogen concentrations at slaughter and improve meat quality. However, when fed, these substrates would be fermented in the rumen to volatile fatty acids. Garza and Owens (1989) reported that 60% of drinking water evaded the rumen of heifers fed roughage diets, whereas the bypass was greater (80%) for heifers fed concentrate diets. Therefore, gluconeogenic substrates supplemented via drinking water may escape microbial fermentation in the rumen and be absorbed in the small intestine to be available for utilization by the tissues. Supplementation of ruminants prior to slaughter with glycerol or fructose as a source of gluconeogenic substrates may improve muscle and liver glycogen concentrations at slaughter and meat quality.

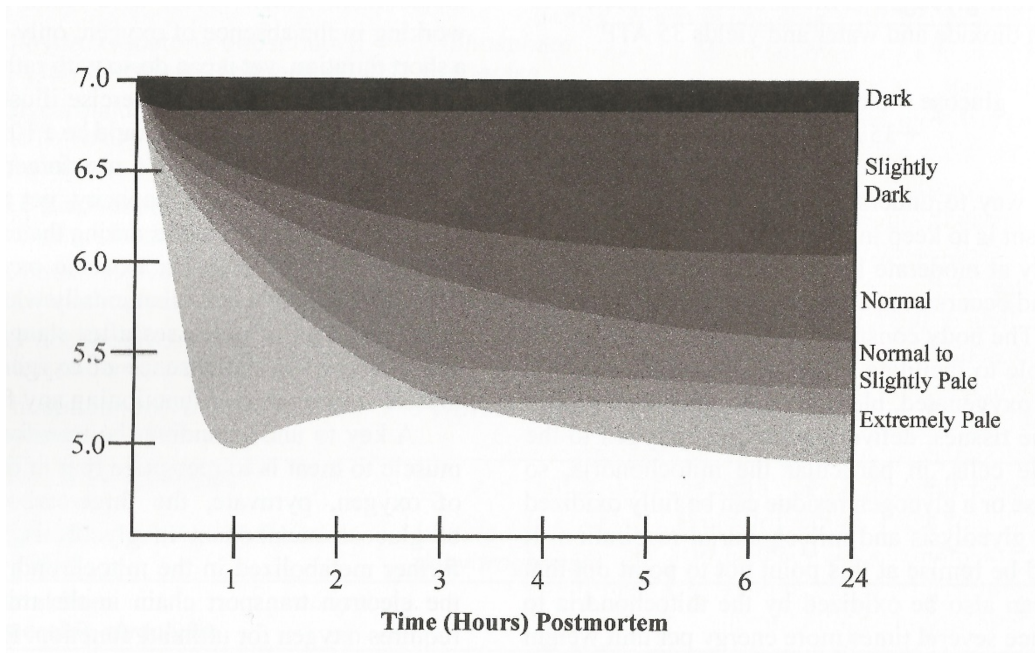

Figure 1.1. Various postmortem pH decline curves observed in pork muscle.

Shaded reflects differences in fresh meat pinkness. From Aberle et al. (2012).

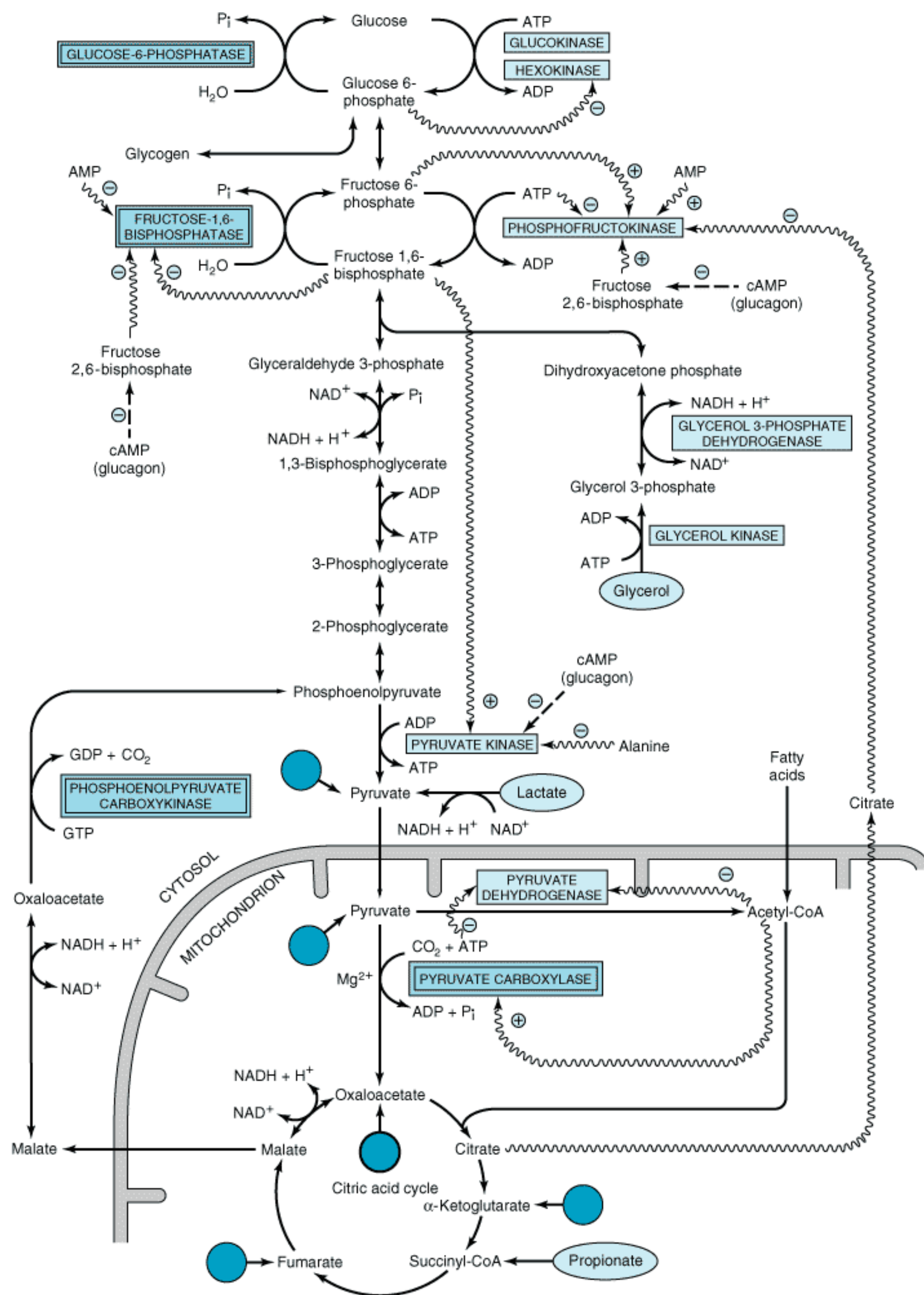

Figure 1.2. Major pathways and regulation of gluconeogenesis and glycolysis in the liver. Entry points of glucogenic amino acids after transamination are indicated by arrows extended from circles. From Bender and Mayes (2012c).

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CHAPTER TWO

SUPPLEMENTATION OF GLYCEROL OR FRUCTOSE VIA DRINKING WATER TO ENHANCE MARBLING DEPOSITION AND MEAT QUALITY OF FINISHING CATTLE

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ABSTRACT

Thirty-six Angus-cross steers (667 ± 34.4 kg initial BW, 24.5 mo) were used to assess the impact of short-term glycerin or high fructose corn syrup administration via drinking water on meat quality and marbling deposition. Steers blocked by BW (3 blocks), were assigned randomly to one of three drinking water treatments: 1) control (CON), 2) 4.3% crude glycerin (GLYC), or 3) 4.3% high fructose corn syrup (HFCS) for the final 25 d prior to slaughter. Average daily gain was lower ($P = 0.01$) and final live weight was lower ($P < 0.01$) with HFCS administration compared to CON. Dry matter intake and water intake did not differ among treatments. Fat thickness, muscle depth and intramuscular fat measured by ultrasound did not differ between treatments. Crude glycerin or HFCS via water supplementation did not alter hot carcass weight, dressing percentage, ribeye area, fat thickness, KPH, skeletal maturity or marbling score. *Longissimus* muscle and subcutaneous fat color (L*, a* and b*) were not affected by

drinking water treatment. Total lipid content, total fatty acid content, and fatty acid composition of the *Longissimus* muscle (LM) did not differ among drinking water treatments. Supplementation of drinking water with GLYC or HFCS did not alter Warner Bratzler shear force values or water holding capacity (drip loss, cook shrink). Intramuscular mean adipocyte diameter was greater (P = 0.02) for steers offered HFCS compared to GLYC with CON being intermediate. These differences in mean adipocyte size were related to changes in the adipocyte size distribution. There were greater proportions of small (20-30 μm) adipocytes in GLYC compared to HFCS and CON. In contrast, HFCS and CON had greater proportions of medium (40-50 μm) adipocytes than GLYC. The relative mRNA expression of lipogenic genes (acetyl Co-A carboxylase [ACC], fatty acid binding protein 4 [FABP4], fatty acid synthase [FASN], glycerol-3-phosphate acyltransferase [GPAT], retinol-binding protein 4 [RBP4], and stearoyl-CoA desaturase [SCD]), adipocyte differentiation genes (delta-like 1 homolog [DLK1]) and transcription factors (CCAAT/enhancer-binding protein α [CEBPα], and PPARγ) was similar for GLYC and HFCS compared with CON. *Longissimus* glycogen and lactate concentrations and glycolytic potential were not affected by drinking water treatments. Overall, HFCS or GLYC supplementation via drinking water did not alter carcass or meat quality variables but did alter the size and distribution of intramuscular adipocytes. These results indicate that a longer supplementation time or a higher substrate level may be needed in order to obtain differences in meat quality.

INTRODUCTION

Ruminants ferment dietary carbohydrates in the rumen into volatile fatty acids that provide over 90% of energetic needs. Compared with that of nonruminants, the relative amount of glucose absorbed is small, although certain tissues are obligatory users of glucose. Therefore, ruminants rely on gluconeogenesis, the process of synthesizing glucose or glycogen from noncarbohydrate precursors. Glucose is an important substrate for intramuscular fat (IMF) synthesis whereas acetate is used for subcutaneous fat synthesis (Smith and Crouse, 1984). Glycogen plays a crucial role in post-mortem pH decline because it is the primary substrate for lactic acid production in muscle. If the concentration of glycogen in the muscle is low and the ultimate pH is high, meat color, water holding capacity and tenderness may be affected adversely (Lowe et al., 2004). Thus supplemental available substrates for IMF and glycogen deposition absorbed from the small intestine may increase marbling and glycogen concentration in the muscle that should enhance carcass value and meat quality.

Crude glycerin, a by-product of the biodiesel industry, can be utilized in livestock diets. Crude glycerin contains approximately 80% glycerol but this level can vary depending on its source. Glycerol, the three-carbon backbone of triglyceride, is a substrate for gluconeogenesis. On the other hand, high fructose corn syrup produced from corn milling has become the leading sweetener utilized in the beverage industry. High fructose corn syrup, at 42% glucose and 55% fructose, can be refined further to enhance fructose levels. High intakes of fructose can lead to increased lipogenesis and fatty acid synthesis (Samuel, 2011; Crescenzo et al., 2014). However, if the glycerol or fructose is

fed to ruminants and enters the rumen, it is fermented to volatile fatty acids and little rumen escape of these substrates to the tissues would occur. Garza and Owens (1989) estimated that 60-80% of drinking water bypasses the rumen. They found that rumen escape of drinking water by beef heifers was greater with high concentrate diets (80%) than with hay diets (62%). Therefore, drinking water may provide a means to deliver substrates to the small intestine of finishing animals for direct absorption and utilization by the tissues. The objective of this research was to supply substrates for gluconeogenesis and/or *de novo* fatty acid synthesis via drinking water to stimulate marbling deposition and enhance meat quality of finishing cattle.

MATERIALS AND METHODS

The experimental procedures were reviewed and approved by Clemson University Animal Care and Use Committee.

Animals. Steers (422 kg) were obtained from Virginia Tech Shenandoah Valley Agricultural Research and Extension Center. Steers were fed several adjustment rations until they reached the final finishing diet (see Table 2.1). After 50 d on feed, steers were weighed and ultrasound was used to measure *Longissimus* muscle (LM) depth, intramuscular fat content and subcutaneous fat thickness. Steers averaged 537 kg BW with an estimated 6.12 cm LM depth, 3.99% intramuscular fat content and 6.8 mm subcutaneous fat thickness.

Four weeks prior to the start of the water supplementation period, steers were blocked by BW (3 blocks) and assigned randomly to one of three drinking water treatments (4 animals/pen; 3 pens/treatment): 1) control (CON), 2) 4.3% crude glycerin (GLYC), or 3) 4.3% high fructose corn syrup (HFCS) and offered the same high concentrate diet (Table 2.1). During this 4-week time period, water supplements were not provided but water intake was recorded daily and dry matter intake was recorded on four consecutive days each week. Water and dry matter intakes averaged 38.4 L/hd/d and 17.3 kg/hd/d, respectively (Table 2.2). These drinking water measurements were used to calculate the amounts of GLYC and HFCS to add to the drinking water during the 25 d test period. Calculations were made based on published glucose turnover rates for beef heifers (Harmon et al., 1983) and adjusted to a metabolic body weight basis (9.12 g glucose/kg$^{0.75\ BW}$). We estimated that 1.1 kg glucose per steer daily would equal the daily glucose turnover. Estimates from the suppliers were that crude glycerin contained 80% glycerol (Glycerin Traders, IN) and high fructose corn syrup was about 80% glucose + fructose (Indiana Sugars, IN). In addition, crude glycerin also had a very low methanol content according to its supplier which is important for a drinking water supplementation. High fructose corn syrup was purchased in 200 L drums and maintained at 18°C using a drum heater to keep it liquid. During the study, daytime temperatures remained above 17°C. High fructose corn syrup water solutions remained mixed in the drinking water as indicated by daily sampling. Garza and Owens (1989) estimated that 80% of drinking water would escape rumen fermentation when beef heifers were fed a high concentrate diet. We adjusted the final concentrations in the drinking water for the percentage

substrate present and this estimated percentage escape from the rumen. Therefore, the target intake was of 1.7 kg per steer daily of crude glycerin or high fructose corn syrup, this equals 4.3% crude glycerin or high fructose corn syrup in drinking water based on expected water intake.

At the start of the 25-d water supplementation period, steers (666.7 ± 34.4 kg, 24.5 mo) were allowed free choice access to the drinking water treatments for 25 d. At this time, steers had been fed the high concentrate for about 80 d. Based on previous research (Duckett et al., 1993), the largest change in intramuscular fat deposition of yearling steers occurs between 84 and 112 d on feed. Drinking water containing GLYC or HFCS were mixed daily; refusals were measured. To be able to measure the intake of water during the experiment, we offered 40% more water than the intake estimated from pre-treatment measures. Water samples were taken daily for determination of glycerol or fructose and glucose. During the experiment, drinking water was recorded daily to determine the total amount of substrate consumed via water.

Steers were weighed and ultrasound measures were taken (Biosoft Toolbox, Biotronics Inc., Ames, IA) weekly for estimation of LM depth, IMF content and subcutaneous fat thickness during the 25-d treatment period. After 25 d on treatments, steers were slaughtered at a commercial packing plant. Steers were stunned by penetrating captive bolt and killed by exsanguination. At 24 h postmortem, carcasses were graded by trained personnel and a rib section (IMPS 107) encompassing the 6th to 12th ribs from each left side of the carcass was identified, removed and transported to the Clemson University Meat Laboratory.

Instrumental color and pH. At 32 h postmortem, color measurements were determined of the exposed LM and subcutaneous fat at the posterior (12th rib) of the rib section. The CIE Lab System was implemented and color measurements were recorded for L* (measures darkness to lightness; lower L* value indicates a darker color), a* (measures redness; higher a* value indicates a redder color), and b* (measures yellowness; higher b* value indicates more yellow color) using a Minolta chromameter (CR-310, Minolta Inc., Osaka, Japan) with a 50-mm-diameter measurement area using a D65 illuminant. The instrument was calibrated using the ceramic disk provided by the manufacturer. Color values were recorded at three locations of each tissue to obtain a representative reading. Muscle pH was measured using a pH meter (Model IQ150, IQ Scientific Instruments Inc., Carlsbad, CA) by inserting the electrode into the core of the LM section parallel to the fibers.

Proximate composition. One steak (2.54 cm thick) from the 12th rib trimmed of all external fat and epimysial connective tissue, was vacuum packaged for proximate composition and fatty acid profile analysis. Steaks were chopped (Blixer®3 Series D, Robot Coupe Inc., Ridgeland, MS) to reduce particle size. Duplicate samples of 5 g were removed for moisture content determination by weight loss by drying at 100°C for 24 h. The remaining samples were frozen at -20°C, lyophilized (VirTis, SP Scientific, Warminster, PA), ground (Blixer®3 Series D), and stored at -20°C for further analyses. Total lipids were extracted from freeze dried samples using an Ankom XT15 extractor (Ankom Technologies, Macedon, NY) with hexane as the solvent. Nitrogen content was determined by the combustion method using a Leco FP-2000 N analyzer (Leco Corp., St.

Joseph, MI). Crude protein was calculated multiplying nitrogen content by 6.25. Total ash content was determined as residual weight after 600°C for 8 h (AOAC, 2000).

Fatty acid profile. Freeze dried samples were transmethylated according to Park and Goins (1994). Fatty acid methyl esters (FAME) were analyzed using an Agilent 6850 (Agilent, San Fernando, CA) gas chromatograph equipped with an Agilent 7673A (Hewlett-Packard, San Fernando, CA) automatic sampler. Separation of FAME was accomplished using a Supelco 100-m SP2560 (Sigma-Aldrich, St. Louis, MO) capillary column (0.25 mm i.d. and 0.20 µm film thickness) using hydrogen as the carrier gas at a flow rate of 1 mL/min. Column oven temperature was programmed to increase from 150 to 174°C at a rate of 2°C/min, from 174 to 178°C at 0.2°C/min, from 178 to 225°C at 2°C/min, and then held at 225°C for 8.5 min. The injector and detector were maintained at 250°C. Sample injection volume was 1 µl. Samples were run twice with a split ratio of 100:1 for *trans* C18:1 and long chain fatty acids and again at split ratio of 10:1 for CLA and omega-3 fatty acids. Individual fatty acids were identified by comparing relative retention times with fatty acid standards (Sigma-Aldrich; Matreya, Pleasant Gap, PA). Fatty acids were quantified by incorporating methyl tricosanoic (C23:0) acid as an internal standard into each sample after methylation; values are expressed as g/100 g of total fatty acids.

Warner-Bratzler Shear Force. Five steaks (8th to 11th ribs) were removed and assigned randomly to postmortem aging lasting 2, 4, 7, 14 and 21 d. Steaks were vacuum packaged, stored at 4°C for their assigned postmortem aging times and then stored frozen at -20°C for Warner-Bratzler shear force analysis. Aged steaks were thawed for 18 h at

4°C and broiled on Farberware (Bronx, NY) electric grills to an internal temperature of 71°C (AMSA, 2015). After steaks cooled to room temperature, six 1.27-cm-diameter cores were removed from each steak parallel to the longitudinal orientation of the LM fibers. All cores were sheared perpendicular to the long axis of the core using a Warner-Bratzler shear machine (G-R Manufacturing, Manhattan, KS).

Water holding capacity. Three adjacent steaks (approximately 100 g) from each rib section were used to measure drip loss using the Honikel bag method (Honikel, 1998). Each steak was weighed, suspended inside a plastic bag and stored at 4°C. At 2, 4, 6 and 9 days, steaks were removed from the bag, dabbed dry with a paper towel and weighed. Drip loss was calculated as the weight difference before and after storage expressed as a percentage of the original weight of the sample before storage. Cooking shrink was measured for the steaks used for Warner Bratzler shear force determination. The steaks were weighed before and after cooking. Shrink loss was calculated as the difference in weight before versus after cooking expressed as a percentage of the raw weight before cooking.

Cell size. One steak from each rib was used for determination of intramuscular adipocyte cell size. Samples also were collected from subcutaneous fat at the 12th rib and intermuscular fat at the 12th rib for adipocyte cell size measurements. Intramuscular fat removed from the LM, subcutaneous fat, and intermuscular fat samples were used for determination of adipocyte cell size according to Etherton et al. (1977). Adipocytes were counted and sized using a particle sizing and counting analyzer (Multisizer 4 Coulter Counter, Beckman Coulter Inc., Brea, CA).

Muscle metabolic substrates/products. One steak from each rib section, trimmed of all external fat and epimysial connective tissue, was chopped, flash frozen in liquid nitrogen and stored at -80°C. For glucose and glucose-6-phosphate, duplicate 1.5 g samples were homogenized in 20 mL 2 *N* hydrochloric acid, incubated 20 min at 4°C, centrifuged at 2630 *x g* for 15 min at 4°C, filtered through Whatman paper and incubated 2 h at 90°C. Samples were cooled in an ice bath and an aliquot (10 mL) was neutralized with 2 *N* sodium hydroxide. The resulting hydrolyzed glycogen as glucose and glucose-6-phosphate were measured using a commercial kit from Sigma (Glucose (HK) assay kit) that is based on coupled enzyme reactions catalyzed by hexokinase and glucose-6-phosphate dehydrogenase. The increase in absorbance at 340 nm was measured in a 96-well plate using a microplate reader spectrophotometer (Synergy HT, BioTek Instruments, Inc.). For lactate, duplicate 3 g samples were homogenized in 10 mL 1.5 *N* perchloric acid, 5 mL of distilled water at 95°C was added and tubes were incubated for 2 min at 90°C. Samples were cooled for 45 min at 4°C and filtered through Whatman paper. An aliquote (10 mL) was neutralized with 2 *N* sodium hydroxide. Lactate concentration was measured in diluted samples (1:4 in water) using a commercial kit (L-Lactate [LAC] kit) from Randox (Kearneysville, WV) that is based on coupled enzyme reactions catalyzed by lactate oxidase and peroxidase. The increase in absorbance at 550 nm was measured in a 96-well plate using a microplate reader spectrophotometer (Synergy HT, BioTek Instruments, Inc.). Glycolytic potential calculations were performed according to Monin and Sellier (1985) where glycolytic potential = [lactate] +

2 ([glycogen] + [glucose-6-phosphate] + [glucose]), and was expressed as millimoles of lactate per kg of muscle.

Gene expression. Total cellular RNA (tcRNA) was extracted from subcutaneous adipose tissue (Trizol, Invitrogen, Carlsbad, CA; PureYield, Promega, Madison, WI), converted to cDNA (Superscript III; Invitrogen), and analyzed by qPCR (QuantiTect SYBR Green PCR kit, Qiagen, Valencia, CA) for relative gene expression according to Duckett et al. (2009). Primers for genes of interest were designed on or spanned exon boundaries, when possible, using Primer3 software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/, accessed 05 August 2014; Table 2.3). Primer sequences for acetyl Co-A carboxylase (ACC), CCAAT/enhancer-binding protein-α (C/EBPα), stearoyl-CoA desaturase (SCD1), peroxisome proliferator-activated receptor-γ (PPAR-γ), glyceraldehyde 3-phosphaste dehydrogenase (GAPDH), and fatty acid synthase (FASN) have been reported previously (Duckett et al., 2009). Primer sequences for other genes of interest, delta-like 1 homolog (DLK1), fatty acid binding protein 4 (FABP4), facilitated glucose transporter 4 (GLUT4), glycerol-3-phosphate acyltransferase (GPAT), and retinol-binding protein 4 (RBP4), are shown in Table 2.3. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene for data normalization. Normalized CT values ($\Delta CT = CT_{,gene} - CT_{,GAPDH}$) were calculated for each sample and subjected to ANOVA, as described below. The fold changes in gene expression for GLY or HFCS vs. CON were calculated by using the $2^{-\Delta\Delta CT}$ method, according to Livak and Schmittgen (2001).

Glycerol concentration. Glycerol concentration in water samples was determined spectrophotometrically using a commercial kit (Free glycerol determination kit) from Sigma-Aldrich (St. Louis, MO) that is based on coupled enzyme reactions catalyzed by glycerol kinase, glycerol phosphate oxidase and peroxidase. The product of the assay is a quinoneimine dye with an absorbance maximum at 540 nm. The increase in absorbance was measured in a 96-well plate using a microplate reader spectrophotometer (Synergy HT, BioTek Instruments, Inc.). Our laboratory analyses showed that crude glycerin utilized in this study contained 87.3% glycerol.

Fructose and glucose concentration. Water samples diluted 1:1 were analyzed by HPLC (Shimadzu Prominence, Columbia, MD) for glucose and fructose concentrations. Samples were separated using a Supelcogel Ca^{2+} column (Sigma-Aldrich, St. Louis, MO) with water as the mobile phase at 0.5 mL/min. Concentrations of glucose and fructose were determined using photodiode array detector at 190 nm (Shimadzu PDA) and compared to a standard curve for each compound. Our laboratory analyses showed that high fructose corn syrup utilized in this study contained 46.9% fructose and 33.5% glucose.

Statistical Analyses. Data were analyzed as a randomized complete block design using PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). For water and dry matter intakes, pen was used as the experimental unit (3 per treatment), and data were analyzed based in the following model: $Y_{ijk} = \mu + \beta_i + \tau_j + \beta\tau_{ij} + \delta_k + \tau\delta_{jk} + \varepsilon_{ijk}$, where Y_{ijk} is the observed value, μ is the overall mean, β_i is the block effect (i = 1 to 3), τ_j is the treatment effect (j = 1 to 3), $\beta\tau_{ij}$ is the random interaction between block and treatment, δ_k

is the day effect (k = 1 to 25 and 1 to 12, for water intake and dry matter intake, respectively), $\tau\delta_{jk}$ is the interaction between treatment and day, and ε_{ijk} is the experimental error. For animal performance, carcass traits, adipose tissue and *Longissimus* determinations, animal was used as experimental unit (12 per treatment), and data were analyzed based in the following model: $Y_{ijk} = \mu + \beta_i + \tau_j + \beta\tau_{ij} + \alpha_k (\beta_i \tau_j) + \varepsilon_{ijk}$, where Y_{ijk} is the observed value, μ is the overall mean, β_i is the block effect (i = 1 to 3), τ_j is the treatment effect (j = 1 to 3), $\beta\tau_{ij}$ is the random interaction between block and treatment, $\alpha_k (\beta_i \tau_j)$ is the random animal effect within block and treatment (k = 1 to 36), and ε_{ijk} is the experimental error. For water holding capacity, the model also included day and their interaction, whereas for Warner Bratzler shear force the model also included postmortem aging time and their interaction; degree of doneness was included in the model as a covariate. Least square means were generated and separated by Fisher's protected LSD. Significance was determined at P < 0.05. Differences of P > 0.05 to P < 0.10 are discussed as trends.

RESULTS AND DISCUSSION

Animal performance data are shown in Table 2.2. Average daily gain during the 25-d supplementation period was lower (*P* = 0.01) for steers fed HFCS compared to CON; GLYC steers had an intermediate ADG. As a result, final live weight was lower (*P* < 0.01) for HFCS compared to CON. Dry matter intake was not different (*P* > 0.05) among treatments so gain to feed ratio was lower (*P* = 0.04) for HFCS steers compared to

CON steers. Glucose supplemented in the drinking water when compared to glucose in feed also tended to reduce animal performance of steers fed a high concentrate diet (Garza et al., 1992). Parsons et al. (2009) evaluated including crude glycerin in the diet of finishing heifers at varying levels (0, 2, 4, 8, 12 or 16% of diet DM) throughout a 85-d finishing period. They reported that DMI increased as the concentration of glycerin increased; feed efficiency and ADG increased up to an 8% glycerin inclusion in the diet.

Fructose, glucose and glycerol are readily fermentable in the rumen and may alter the fermentation products pattern. Golder et al. (2012) reported that ruminal pH decreased and total VFA and lactate concentrations increased in dairy heifers when part of supplemented grain was substituted for fructose. Wang et al. (2009) observed a linear decreased in ruminal pH and a linear increase in total VFA, propionate and butyrate concentrations in steers with increasing glycerol supplementation. These changes in ruminal fermentation patterns may increase acidosis risk in a high concentrate diet scenario impairing animal performance.

Water intake was not significantly affected ($P > 0.05$) by treatment. The intake of glycerol averaged 1.4 kg per head daily for GLYC animals and was similar to intake of fructose plus glucose (0.82 and 0.58 kg per head daily, respectively) for HFCS. Others have reported no differences in water intake of lactating dairy cows supplemented with 2% glycerin (Osborne et al., 2009) or 2% glucose (Osborne et al., 2002) in drinking water compared with control cows.

Fat thickness, LM muscle depth and IMF measured by ultrasound methods did not differ among treatments (*P* > 0.05, Table 2.2). The rates of subcutaneous fat deposition were similar (*P* > 0.05) between treatments averaging 0.098 mm/d (data not shown). Similarly, IMF deposition rate did not differ (*P* > 0.05) among treatments averaging 0.054 percentage points per day. Intramuscular fat percentages during the study measured by real-time ultrasound are shown in Figure 2.1. Values for IMF did not differ between treatments (*P* > 0.05) at any time point. Intramuscular fat values increased during the 25-d finishing period (about 1.13% units), but changes in IMF appear greater prior to the start of the water supplementation period (pre-scan at -56 d to 0 d, 2.67%). Duckett et al. (1993) previously reported that total lipid content doubled between 84 and 112 d on a high-concentrate diet but that neither marbling scores nor total lipid content increased after 112 d. Supplementation of the drinking water treatments might have proven more beneficial earlier during finishing, but extreme weather throughout the US delayed arrival of substrates to the test site and low ambient temperatures (high temp < 13°C; with night time freezing) caused HFCS to segregate in the drinking water solution.

Hot carcass weight, dressing percentage, ribeye area, fat thickness, skeletal maturity, KPH, and marbling score were not affected significantly by treatments (*P* > 0.05, Table 2.4). The percentage of cattle grading USDA Choice or greater was 92%. These results agree with those of Parsons et al. (2009) who fed crude glycerin in the diet of finishing heifers at various levels (0, 2, 4, 8, 12 or 16% of diet DM) throughout 85-d finishing period and found no improvements in carcass parameters. Others (Schaefer et al., 1990), observed a greater dressing percentage in yearling bulls supplemented with 5%

glucose in drinking water during lairage compared to animals receiving only water. They found that glucose treatment for 18 to 20 h before slaughter reduced carcass shrink by 3%. These improvements in carcass yield coincided with enhanced electrolyte balance for the bulls in the glucose-water supplementation treatment.

Longissimus muscle color (L*, a* and b*) was not affected ($P > 0.05$) by treatment. Similarly, there were no effects ($P > 0.05$) of treatment on subcutaneous fat color (L*, a* and b*). Proximate composition also was similar ($P > 0.05$) among treatments (Table 2.5). Total lipid content was not affected ($P > 0.05$) by treatment (Table 2.5). The fatty acid profile of LM was similar ($P > 0.05$) between animals (Table 2.5). Numeric values agree with *Longissimus* profiles reported for cattle finished on high concentrate diets (French et al., 2000; Realini et al., 2004).

Intermuscular and subcutaneous fat cell diameters were not affected ($P > 0.05$) by treatments (Table 2.4). However, intramuscular fat cell diameter was greater ($P = 0.02$) for steers offered HFCS compared to GLYC whereas CON steers had intramuscular fat cells with an intermediate diameter (Table 2.4). Changes in mean diameter appear related to an alteration in distribution of intramuscular adipocyte size. The percentage of intramuscular adipocytes between 20 and 30 μm was higher for GLYC compared to HFCS and CON (Figure 2.2); in contrast, HFCS and CON steers had higher percentages of 40-50 μm adipocytes. The increase in small diameter adipocytes with GLYC may indicate that intramuscular adipogenesis was stimulated. Accretion of bovine adipose tissue is the result of both adipocyte hyperplasia (increased cell number) and hypertrophy (increased cell size; Jo et al. 2009). Postnatal fat accretion occurs primarily by filling

existing adipocytes, with a smaller contribution of hyperplasia (Sainz and Hasting, 2000). Robelin (1986) estimated that about 30% of the increased in fat deposition during fattening was due to hyperplasia versus 70% from hypertrophy. However, individual fat depots vary in the timing of hyperplastic and hypertrophic growth (Sainz and Hasting, 2000). Intramuscular fat is a late developing depot in which hyperplasia plays a very important role (May et al., 1994; Yang et al., 2006). The substrates for lipogenesis may also vary between individual fat depots. According to Smith and Crouse (1984), 50-75% of lipogenesis in intramuscular adipose tissue uses glucose as substrate. In contrast, subcutaneous fat uses mainly acetate.

The hepatic metabolism of fructose has important effects of lipid metabolism. Fructose bypasses the main regulatory steps in glycolysis and hepatic triacylglycerol production is facilitated (Elliot et al., 2002). Fructose can provide carbon atoms for both the glycerol and the acyl portions of acyl-glycerol molecules (Mayes et al., 1993). Thus high intakes of fructose can serve as relatively unregulated source of acetyl-CoA (Elliot et al., 2002). Rats fed a fructose-rich diet have been shown to exhibit a significant increase in plasma triglycerides (Crescenzo et al., 2014). Fructose supplementation has been shown to enhance *de novo* fatty acid synthesis and increase body fat stores in rats (Crescenzo et al., 2013) and humans (Stanhope et al., 2009). The lack of change in carcass marbling parameters likely indicates that the supply of glycerol and fructose reaching the small intestine for absorption and utilization was insufficient. This may be due to lower rumen escape of drinking water in this study than observed by Garza and Owens (1989) for beef heifers. Additional research would be needed to determine if

longer supplementation and/or higher doses of substrates in drinking water would have a greater impact on marbling deposition. Supplementation of substrates to enhance marbling may be more effective if occurred earlier in life. According to Du et al. (2010), the adipogenic potency gradually declines postnatally because of depletion of multipotent cells in skeletal muscle, thus nutritional supplementation becomes less effective in increasing the number of intramuscular adipocytes although the size of existing intramuscular adipocytes can be increased during fattening. The relative mRNA expression of lipogenic genes (ACC, FABP4, FASN, GPAT, RBP4 and SCD), and transcription factors (CEBPα, and PPARγ), did not differ ($P > 0.05$) for GLYC and HFCS as compared with CON (Figure 2.3). Others have shown that high fructose diets induced higher mRNA expression of SCD1, ACC and FASN in mouse liver (Miyazaki et al., 2004) and hepatic ACC and FASN in rat (Janevski et al., 2012). Robubi et al. (2014) found increases in FASN mRNA levels in cultured human adipocytes incubated with fructose and glucose as compared to cells incubated with only glucose. The relative mRNA expression of facilitated GLUT4, the major insulin-dependent glucose transporter in adipocytes, was similar ($P > 0.05$) for GLYC and HFCS compared with CON (Figure 2.3). Robubi et al. (2014) reported an up-regulation of GLUT4 in cultured human adipocytes supplemented with fructose.

Warner Bratzler shear force (Figure 2.4) was not affected ($P = 0.63$) by supplementation treatments. However, shear force values decreased ($P < 0.0001$) as postmortem aging days increased. Miller et al. (2001) suggest a guaranteed-tender threshold of < 3.0 kg for customer satisfaction. In our study, 7 days of postmortem aging

was sufficient to reach this average WBS value. Water holding capacity, determined as drip loss (Figure 2.5) and cook shrink (Table 2.5), was not affected by treatment ($P > 0.05$). Glycerol has been used successfully as an osmotically active agent in hyperhydration for increased fluid retention in humans (van Rosendal et al., 2010; Ross et al., 2012) and horses (Schott et al., 2001). Early postmortem events including rate and extent of pH decline and proteolysis are key to influence the ability of meat to retain water (Huff-Lonergan and Lonergan, 2005) due its influence on protein to protein interactions (Hamm, 1975). In our study, no differences in pH between treatments were significant ($P > 0.05$, Table 2.4). Drip loss increased ($P < 0.0001$) with postmortem storage time (Figure 2.5). These results agree with other studies (Mitsumoto et al., 1995; den Hertog-Meischke et al., 1997). Several authors (Kristensen and Purslow, 2001; Huff-Lonergan and Lonergan, 2005; Farouk et al., 2012) indicate that water holding capacity of meat with storage is improved by greater degradation of cytoskeleton proteins during postmortem aging. Degradation of cytoskeleton slowly removes the linkage between lateral shrinkage of myofibrils and shrinkage of entire muscle fibers, so removing the force that causes flow into the extracellular space (Kristensen and Purslow, 2001).

Longissimus glycogen/glucose and lactate concentrations, as well as glycolytic potential were not affected ($P > 0.05$) by treatment (Figure 2.6). These values fell within the range reported by Immonen and Puolanne (2000) and Wulf et al. (2002) for beef with normal ultimate pH. The normal pH reported in our study (Table 2.4) would indicate that the glycogen content in the muscle at the moment of slaughter was sufficient for fueling lactic acid production, thus enabling pH to decline. Several studies have indicated that

muscle glycogen concentration largely reflects daily metabolizable energy intake (Immonen et al., 2000; Gardner at al., 2001a). Lack of effect of treatment on muscle glycogen concentration in our study may be due to the high levels of muscle glycogen associated with high concentrate diets. Lambs finished on grain-based feedlot rations had higher muscle glycogen concentrations than lambs finished on pasture (Jacob et al., 2005). This may be due to greater ruminal production of propionate, a gluconeogenic substrate for ruminants fed concentrate diets. Gardner et al. (2001b) reported increased plasma glucose concentrations when sheep fed low or high energy diets were offered water containing 3.5% glycerol and 1.5% propylene glycol over a 24 h period. The authors reported that the hyperglycemic effect was greater for roughage-fed animals; this may reflect a greater effect of such supplements by pasture-fed than by concentrate-fed animals.

In general, effects of glycerol or high fructose corn syrup supplementation via drinking water had little or no impact on performance and meat quality except for changes in intramuscular adipocyte size. These results indicate that our substrates likely did not reach the small intestine in significant amounts or were not supplemented for a sufficiently long time period, or this outcome be due to less bypass of the drinking water supplements than estimated by Garza and Owens (1989) using beef steers. In lactating dairy cows, Woodford et al. (1984) reported that only 18% of ingested water bypassed the rumen. Additional research is needed to determine if supplementing glycerol or high fructose corn syrup in drinking water to increase postruminal substrates can increase intramuscular lipid deposition.

Table 2.1. Composition of finishing ration fed to steers during the experiment.

Ingredient composition	
Cracked corn, %	81.20
Bermudagrass hay, %	10.00
Soybean meal, %	6.70
Limestone, %	1.85
Salt, %	0.25
Proximate analysis[1]	
Dry matter, %	87.74 ± 0.16
Crude Protein, % DM	10.02 ± 1.00
NDF, % DM	26.40 ± 1.36
ADF, % DM	11.34 ± 0.48
Ash, % DM	3.92 ± 0.46

[1]Based on feed samples taken each week.

Table 2.2. Animal performance, dry matter and water intake, and ultrasound measurements of steers offered crude glycerin (GLYC) or high fructose corn syrup (HFCS) in drinking water or regular drinking water (CON).

	GLYC	HFCS	CON	SEM	P-level
Pre-trial period (4 wk prior)					
Average daily gain, kg/d	2.25	2.29	2.59	0.265	0.641
Water intake, L/hd/d	39.51	38.55	37.67	1.457	0.694
Dry matter intake, kg/hd/d	15.0	15.3	15.7	0.39	0.479
Trial period (25 d)					
Average daily gain, kg/d	1.77[a,b]	1.52[b]	1.96[a]	0.070	0.029
Final live weight, kg	703.0[a,b]	694.3[b]	712.9[a]	2.71	0.021
Dry matter intake, kg/hd/d	13.6	13.9	14.4	0.31	0.300
Feed efficiency, kg gain/ kg DMI	0.130[c]	0.115[d]	0.136[c]	0.005	0.089
Water intake, L/hd/d	37.69	40.46	36.55	1.618	0.318
Glycerol intake, kg/hd/d	1.4	-	-		
Glucose intake, kg/hd/d	-	0.58	-		
Fructose intake, kg/hd/d	-	0.82	-		
Ultrasound measurements					
Initial fat thickness, mm	11.6	11.0	12.6	1.22	0.659
Initial muscle depth, cm	7.14	6.86	6.92	0.274	0.773
Final fat thickness, mm	13.6	12.8	15.0	0.79	0.248
Final muscle depth, cm	7.36	7.32	7.36	0.300	0.994

[a,b]Means in a row followed by different superscripts are significantly different ($P < 0.05$).

[c,d]Means in a row followed by different superscripts tended to differ ($P < 0.10$).

Table 2.3. Primer sequences (5' to 3') for quantitative real-time PCR.

Gene[1]	Forward	Reverse
DLK1	TCTGCGCTACAACCACATGT	TGGTGAAGGTGGTCATGTCG
FABP4	CATCTTGCTGAAAGCTGCAC	AGCCACTTTCCTGGTAGCAA
GLUT4	ACCTTATGGCCACTCCTCCT	CTCAGCCAACACCTCAGACA
GPAT	TCCCCGACACTGATAAGGAG	GGAGCTTCTGGTGCTTGAAC
RBP4	CCGAGTCAAGGAGAACTTCG	TGCACACACGTCCCAGTTAT

[1]DLK1: delta-like 1 homolog (Drosophila); FABP4: fatty acid binding protein 4; GLUT4: facilitated glucose transporter member 4; GPAT: glycerol-3-phosphate acyltransferase; RBP4: retinol-binding protein 4.

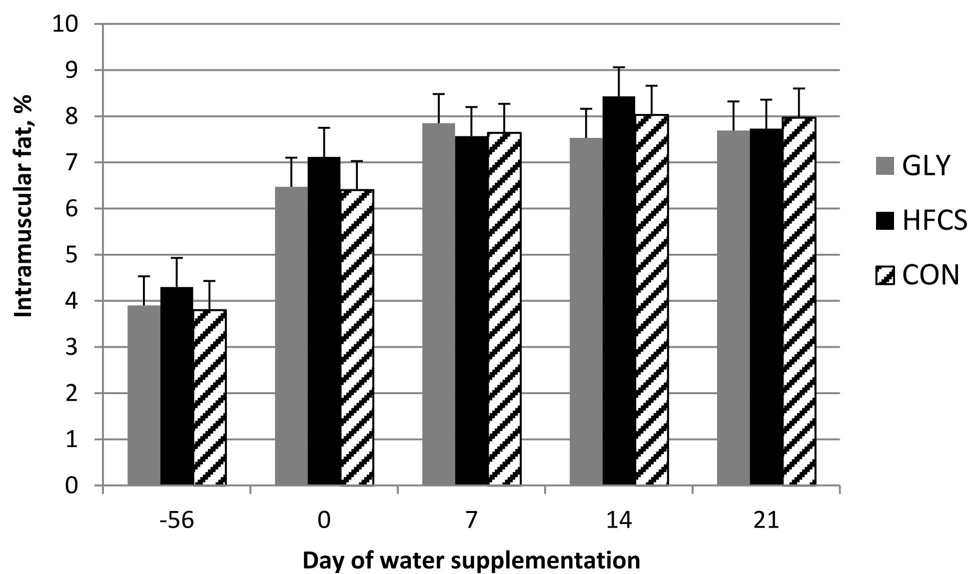

Figure 2.1. Ultrasound intramuscular fat of steers supplemented with crude glycerin (GLYC), high fructose corn syrup (HFCS) or nothing (CON) via drinking water for 25 d.

Table 2.4. Carcass traits, instrumental color, and adipocyte size of steers offered crude glycerin (GLYC) or high fructose corn syrup (HFCS) in drinking water or regular drinking water (CON).

	GLYC	HFCS	CON	SEM	P-level
Hot carcass weight, kg	408.2	405.2	412.1	2.62	0.286
Dressing percentage, %	61.8	62.2	61.6	0.32	0.515
Ribeye area, cm^2	85.9	82.9	85.9	2.63	0.677
Skeletal maturity[1]	179	178	177	1.6	0.588
Fat thickness, mm	14.6	14.4	16.2	0.83	0.345
KPH[2], %	2.8	2.7	2.9	0.08	0.300
Yield grade	3.65	3.74	3.85	0.185	0.764
Marbling Score[3]	597	601	618	28.8	0.858
LM[4] pH	5.53	5.50	5.51	0.019	0.493
LM L*	43.7	44.5	44.8	0.34	0.188
LM a*	29.3	30.0	30.1	0.49	0.484
LM b*	11.2	11.8	11.8	0.22	0.177
s.c.[5] L*	77.4	78.2	77.5	0.84	0.765
s.c. a*	15.8	14.6	16.2	1.01	0.560
s.c. b*	18.1	17.5	17.3	0.37	0.333
Intramuscular adipocyte, µm	47.0^{b}	51.2^{a}	$49.3^{a,b}$	0.76	0.045
Intermuscular adipocyte, µm	51.4	55.8	52.3	2.38	0.454
Subcutaneous adipocyte, µm	52.1	49.7	50.7	0.76	0.196

^{a,b}Means in a row followed by different letters are significantly different ($P < 0.05$).

[1]Skeletal maturity: 100-199= A.

[2]KPH: kidney, pelvic and heart fat as percentage of carcass weight.

[3]Marbling score: 500= small^0, 600= modest^{0}.

[4]LM: *Longissimus* muscle.

[5]s.c.: subcutaneous fat.

Table 2.5. *Longissimus* muscle proximate composition, fatty acid profile, and cook shrink of steers offered crude glycerin (GLYC) or high fructose corn syrup (HFCS) in drinking water or regular drinking water (CON).

	GLYC	HFCS	CON	SEM	P-level
Proximate composition[1]					
Moisture	71.1	71.2	71.0	0.42	0.938
Total lipids	6.1	5.6	5.9	0.56	0.865
Crude protein	22.7	22.9	23.0	0.16	0.445
Ash	1.0	1.07	1.1	0.030	0.695
Fatty acid profile[2]					
SFA[3], g/100 g	42.84	42.29	42.80	0.357	0.536
MUFA[4], g/100 g	45.55	45.62	46.12	0.400	0.588
PUFA[5], g/100 g	3.37	3.54	3.19	0.218	0.580
PUFA n-6, g/100 g	2.71	2.90	2.59	0.169	0.490
PUFA n-3, g/100 g	0.66	0.64	0.60	0.055	0.774
PUFA n-6/PUFA n-3	4.18	4.66	4.40	0.167	0.250
TFA[6], g/100 g wet basis	4.68	4.61	4.72	0.475	0.987
Cook shrink, %	30.05	30.11	29.81	0.416	0.870

[1]In g/100 g wet basis.

[2]In g/100 g of total fatty acids (TFA).

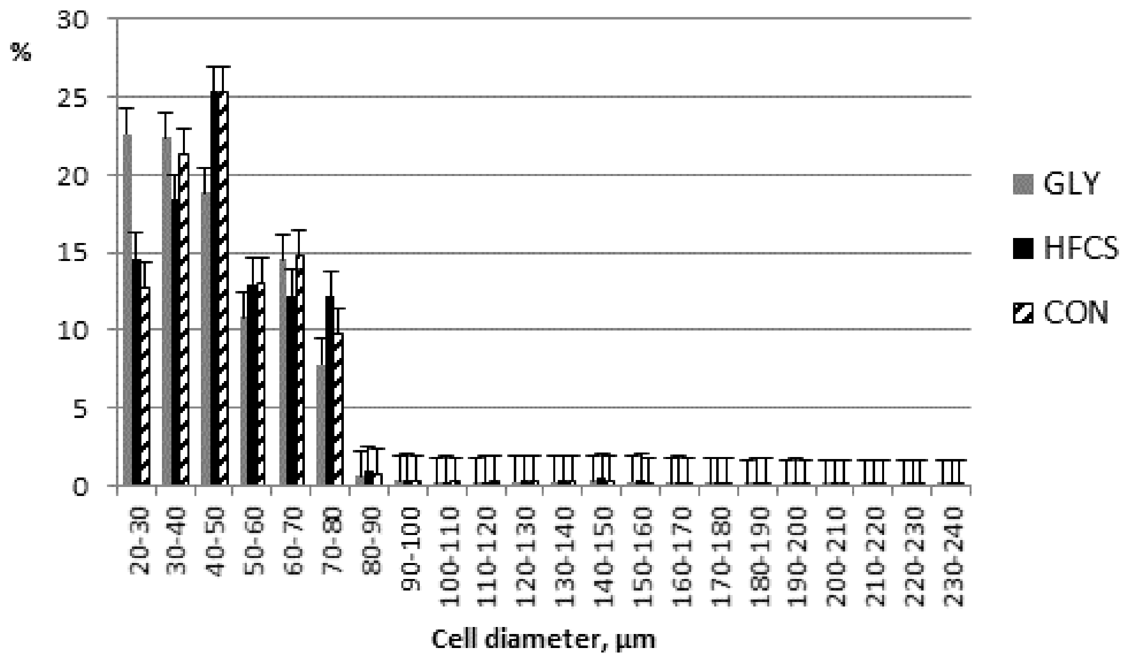

Figure 2.2. Intramuscular adipocyte diameter distribution of steers supplemented with crude glycerin (GLYC), high fructose corn syrup (HFCS) or nothing (CON) via drinking water for 25 d.

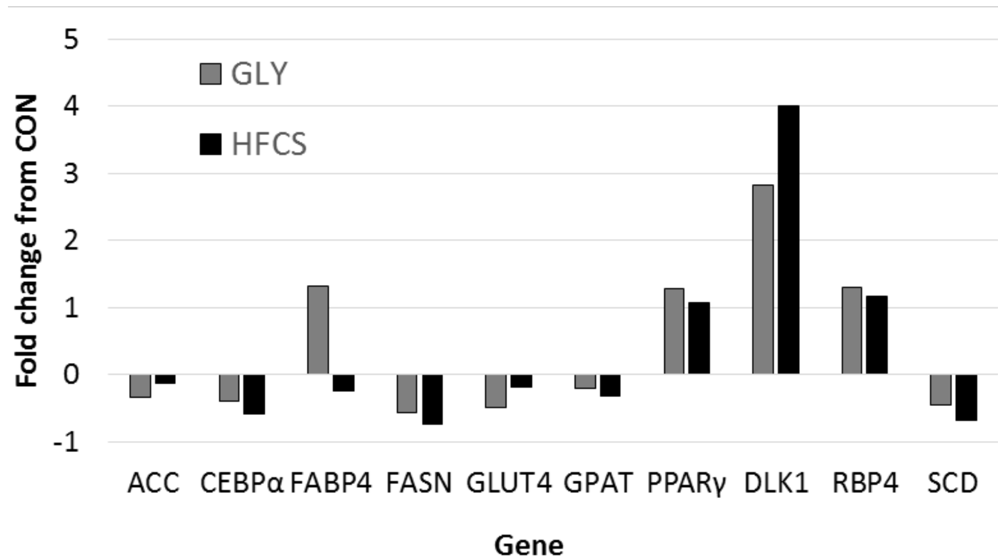

Figure 2.3. Fold change in relative mRNA expression of acetyl-CoA carboxylase (ACC), CCAAT/enhancer-binding protein alpha (CEBPα), fatty acid binding protein 4 (FABP4), fatty acid synthase (FASN), facilitated glucose transporter member 4 (GLUT4), glycerol-3-phosphate acyltransferase (GPAT), peroxisome proliferator-activated receptor gamma (PPARγ), delta-like 1 homolog (Drosophila) (DLK1), retinol-binding protein 4 (RBP4), and stearoyl-CoA desaturase (SCD) in the subcutaneous fat of steers supplemented with crude glycerin (GLYC), high fructose corn syrup (HFCS) or nothing (CON) via drinking water for 25 d. The relative expression of genes for GLYC or HFCS treatments relative to CON were not significant ($P > 0.05$).

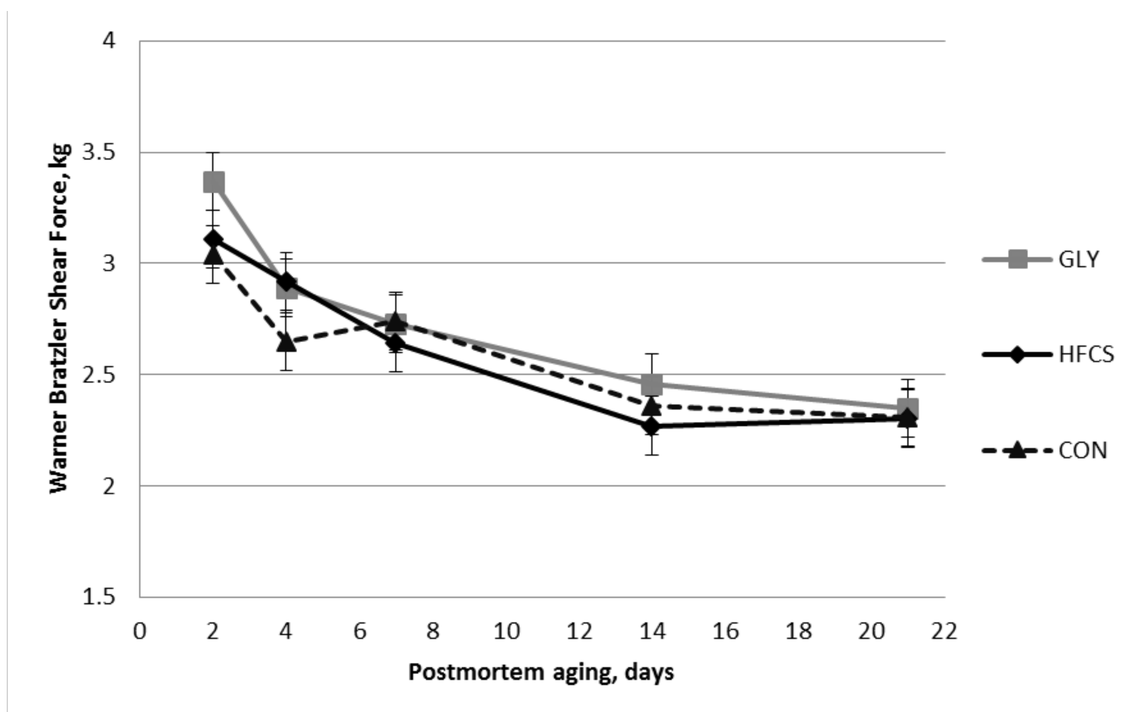

Figure 2.4. Warner Bratzler shear force of steaks aged for 2, 4, 7, 14 or 21 days of steers supplemented with crude glycerin (GLYC), high fructose corn syrup (HFCS) or nothing (CON) via drinking water for 25 d. WBS was not affected by treatments ($P = 0.63$) but was affected by postmortem aging time ($P < 0.0001$). The interaction between treatment and postmortem aging time was nonsignificant ($P > 0.05$).

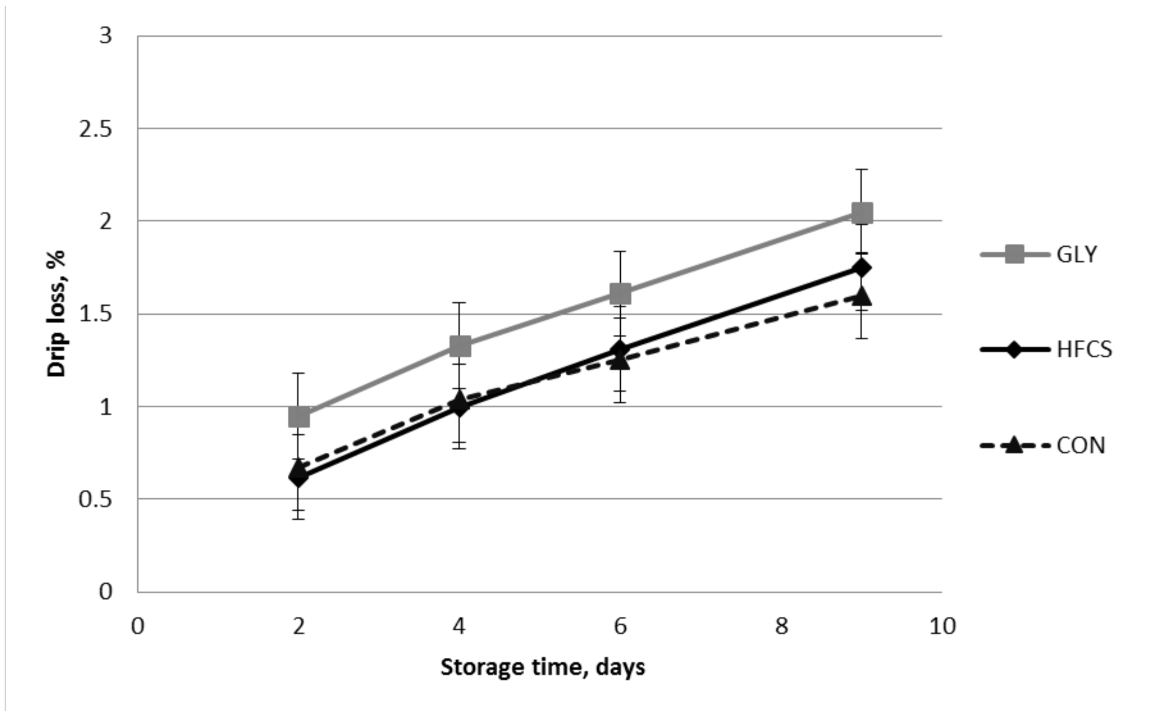

Figure 2.5. Drip loss of steaks storaged for 2, 4, 6, and 9 days of steers supplemented with crude glycerin (GLYC), high fructose corn syrup (HFCS) or nothing (CON) via drinking water for 25 d. Drip loss was not affected by treatments ($P = 0.46$) but was affected by storage time ($P < 0.0001$). The interaction between treatment and storage time was nonsignificant ($P > 0.05$).

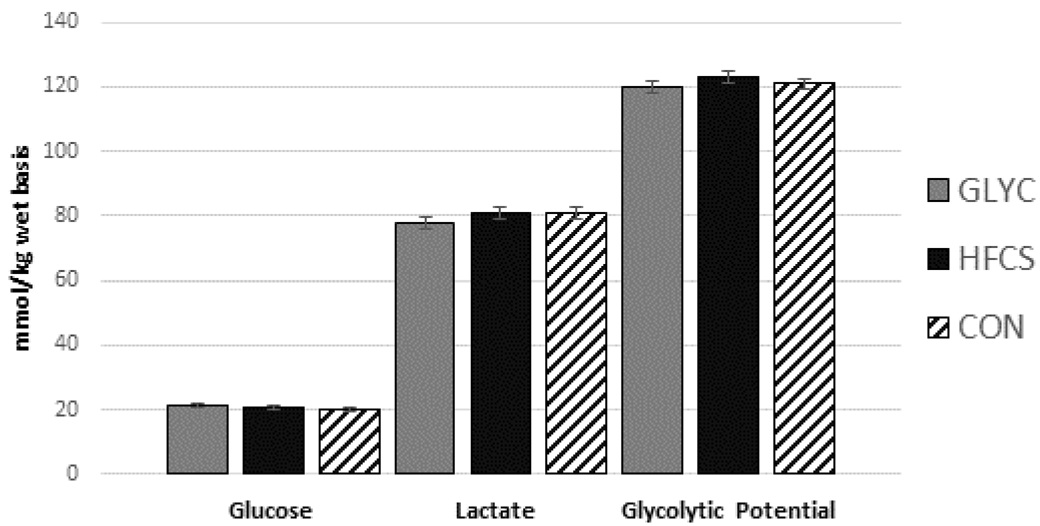

Figure 2.6. Glucose and lactate concentrations, and glycolytic potential 32 h postmortem of steers supplemented with crude glycerin (GLYC), high fructose corn syrup (HFCS) or nothing (CON) via drinking water for 25 d. Glycolytic potential= [lactate] + 2 ([glycogen] + [glucose-6-phosphate] + [glucose]), expressed as milimoles of lactate per kilogram of muscle.

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CHAPTER THREE

SUPPLEMENTATION OF GLYCEROL OR FRUCTOSE VIA DRINKING WATER OF GRAZING LAMBS

ABSTRACT

Eighteen wether lambs (40.1 ± 7.4 kg BW, 4.9 mo.) were used to assess supplementation of glycerol or fructose via drinking water on animal performance, tissue glycogen content and meat quality. Lambs were blocked by BW and allocated to alfalfa paddocks (2 lambs/paddock; 3 paddocks/treatment). Each paddock within block was assigned randomly to drinking water treatments for 30 d: 1) control (CON), 2) 120 g fructose/L (FRU), or 3) 120 g glycerol/L (GLY). Lambs grazed alfalfa for 28 d with free access to drinking water treatments. Then lambs were removed from alfalfa pastures and fasted in indoor pens for 2 d prior to slaughter with access to water treatments only. Data were analyzed using the mixed procedure of SAS. Water intake did not differ ($P > 0.05$) between treatments. During the 28 d grazing period, ADG was greater ($P < 0.05$) for GLY than CON or FRU. During the 2 d fasting period, BW shrink was lower ($P < 0.05$) for GLY (-0.392 kg/an/d) compared to CON (-1.680 kg/an/d) or FRU (-1.340 kg/an/d). Hot carcass weight was greater ($P < 0.05$) for GLY, intermediate for CON and lower for FRU. *Longissimus* muscle (LM) color, water holding capacity, and pH were not affected by treatment ($P > 0.05$). Glycogen content was measured in LM and *Semitendinosus* muscle (ST) at 30 min, 1, 2, 3, 4, 5, 6, 12, and 24 h postmortem. There was a significant treatment by postmortem time interaction ($P = 0.003$). *Longissimus* glycogen content was

greater ($P < 0.05$) for GLY at 2 and 3 h and for FRU at 1 h postmortem compared to CON. Glycogen content in ST did not differ between treatments ($P > 0.05$). Liver glycogen content was greater ($P < 0.05$) for GLY compared to FRU or CON. Liver free glucose was greater for GLY, intermediate for FRU and lower for CON ($P < 0.05$). Liver lipid content was higher for CON, intermediate for FRU and lower for GLY ($P < 0.05$). Serum NEFA was higher ($P < 0.05$) for CON and FRU compared to GLY. Supplementation with GLY increased ($P < 0.05$) odd-chain fatty acid in LM, subcutaneous fat (SQ), and liver. Stearic (C18:0) acid concentrations were reduced in LM ($P = 0.064$) and SQ ($P = 0.018$) whereas oleic (C18:1 cis-9) acid concentration tended to be increased ($P = 0.066$) in SQ with FRU and GLY supplementation. The concentration of linolenic (C18:3 n-3) acid was reduced ($P = 0.031$) and all long-chain n-3 fatty acid (EPA, DPA, DHA) concentrations were increased ($P < 0.05$) with FRU and GLY compared to CON. The mRNA expression of genes associated with lipogenesis (acetyl-CoA carboxylase [ACC], fatty acid synthase [FASN], and stearoyl-CoA desaturase-1 [SCD1]) as well as glucose transport (facilitated glucose transporter member 2 [GLUT2] and facilitated glucose transporter member 4 [GLUT4]) was upregulated in GLY compared to CON. Overall, glycerol supplementation increased ADG during the grazing period, reduced BW shrink during fasting, and increased HCW. Glycerol supplementation favored muscle glycogen at early postmortem times and glycogen content in the liver, decreased lipid mobilization during fastening and upregulated mRNA expression of lipogenic and glucose transport genes in liver.

INTRODUCTION

Glycogen plays a crucial role in post-mortem pH decline since it is the primary substrate for lactic acid production in the muscle. If the glycogen content before slaughter is low, as a result of an insufficient dietary supply or due to long-term stress that causes the depletion of glycogen antermortem, lactic acid accumulation is limited (Warriss, 2000) and the ultimate pH remains high (above 6.0) (Lahucky et al., 1998). Depletion of glycogen prior to slaughter may lead to a higher incidence of dark cutting meats, which are characterized by a high ultimate pH, which adversely affects certain meat quality parameters, such as color, water holding capacity (WHC), and tenderness (Gregory, 2003). Since stress during pre-slaughter handling is an inevitable process, a strategy to mitigate this may be to increase muscle glycogen levels prior to slaughter (Immonen et al., 2000).

Forage-finished animals typically have lower muscle glycogen concentrations and can have higher muscle pH postmortem (McVeigh and Tarrant, 1982; Jacob et al., 2005; Knee et al., 2007). We hypothesize that supplementation of pasture-fed lambs prior to slaughter with glycerol or fructose as gluconeogenic substrates may improve muscle glycogen concentrations at slaughter. However if these substrates were fed directly to ruminants, they would be fermented in the rumen affecting the ruminal environment and as a consequence would alter the characteristics of a pasture-fed animal product. Therefore, we supplemented glycerol and fructose in drinking water as a means to bypass rumen fermentation and increase absorption of these substrates to the intestine. Woodford et al. (1984) and Garza and Owens (1989) estimated that 18-80% of drinking water

bypasses the rumen. Previous research (Volpi-Lagreca and Duckett, 2016) using drinking water substrates to finishing beef cattle suggested that our initial concentrations of 4.3% crude glycerin or high fructose corn syrup altered intramuscular adipocyte size and distribution but that the concentration was too low to alter meat quality parameters. In commercial soda (ex. Mountain Dew), the level of total sugar as analyzed was 12% (fructose + glucose; Ventura et al. 2011) and therefore, we chose to supply substrates in drinking water at this level. The objective of this study was to evaluate the effect of supplementation of glycerol or fructose at 12% via drinking water to grazing lambs on animal performance, muscle glycogen and lactate concentrations postmortem, rate of pH decline, proximate and fatty acid composition of tissues, and changes in gene expression of lipogenic and glucogenic enzymes in liver.

MATERIALS AND METHODS

The experimental procedures were reviewed and approved by Clemson University Animal Care and Use Committee, AUP2015-035.

Animals and treatments. Eighteen Southdown wether lambs (40.1 ± 7.4 kg, 4.9 mo) were used for the study. One week prior to the start of the water supplementation period, lambs were weighed, blocked by body weight (3 blocks) and allocated to alfalfa (*Medicago sativa*) paddocks (2 hd/paddock, 0.125 ha/paddock). Each paddock within block was assigned randomly to one of three drinking water treatments: 1) control, regular drinking water (CON), 2) 120 g fructose per liter of drinking water (FRU), or 3) 120 g glycerol per liter of drinking water (GLY). During this 1-week time period, water

supplements were not provided but water intake was recorded daily and averaged 3.52 ± 1.25 L/hd/d to establish a baseline water consumption level. During the 30-d treatment period, water substrates were offered at the baseline levels plus 40% more for ad libitum consumption of drinking water supplements. Previous research (Volpi-Lagreca and Duckett, 2016) using drinking water substrates to finishing beef cattle suggested that our initial concentrations of 4.3% crude glycerin or high fructose corn syrup were low. Therefore in this study, we utilized a target level of 12% glycerol or fructose in drinking water, which corresponds to the level of total sugars found in commercial soda (Ventura et al., 2011). The substrates used in this experiment were crystalline fructose (generously provided by Tate & Lyle, IL) for FRU and crude glycerin (Glycerin Traders, La Porte, IN) for GLY. The crude glycerin used in this experiment contained 87.3% glycerol; the percentage of crude glycerin was adjusted to provide 12% of glycerol in the drinking water. In addition, crude glycerin had a very low methanol content according to its supplier which is important for a drinking water supplementation. At the start of the 30-d water supplementation period, lambs were allowed free choice access to the drinking water treatments for 30 d. Drinking water containing fructose or glycerol was mixed daily and recorded; refusals were measured. Water intake was adjusted for differences in water volume due to evaporation or rains. To calculate level of substrates intake, samples of drinking water and refusals were taken daily for determination of glycerol or fructose concentration. These data were used to determine the total amount of substrate consumed via water. Pasture availability was measured at the beginning of the supplementation period and after 14 d of grazing, and averaged 857.3 and 1009.1 kg DM/ha, respectively.

Pasture allowance did not constitute a limiting factor. Alfalfa chemical composition is shown in Table 3.1. Lambs remained grazing the alfalfa pasture for 28 d and then were moved to indoor pens at the Clemson University Small Ruminant Research Facility in pens corresponding to paddocks where they had access to the water supplementation treatments but no feed for 2 d fasting period. Lambs were weighed at 0, 14, 28 and 30 d during the treatment period. Ultrasound measures were taken (Biosoft Toolbox, Biotronics Inc., Ames, IA) at the beginning and the end of the study for estimation of subcutaneous fat (SQ) thickness and fat deposition rate. After 30 d on treatments (28 d on pasture and 2 d fasting), lambs were slaughtered at Clemson University Meat Laboratory.

Glycerol concentration in water samples. Glycerol concentrations in water samples were determined spectrophotometrically using a commercial kit (Free glycerol determination kit) from Sigma-Aldrich (St. Louis, MO) that is based on coupled enzyme reactions catalyzed by glycerol kinase, glycerol phosphate oxidase and peroxidase. The product of the assay is a quinoneimine dye with an absorbance maximum at 540 nm. The increase in absorbance was measured in a 96-well plate using a microplate reader spectrophotometer (Synergy HT, BioTek Instruments, Inc.). Concentrations of glycerol were determined by comparison to a standard curve.

Fructose concentration in water samples. Water samples were analyzed by HPLC (Shimadzu Prominence, Columbia, MD) for fructose concentration. Samples were separated using a Supelcogel Ca^{2+} column (Sigma-Aldrich, St. Louis, MO) with water as the mobile phase at 0.5 mL/min. Concentrations of fructose were determined using photodiode array detector at 190 nm (Shimadzu PDA) and compared to a standard curve.

Blood metabolites. Blood metabolites were determined in samples obtained at slaughter. Plasma and serum samples were stored at -20°C until analysis. Glucose concentration was determined in plasma colorimetrically in triplicate using a Glucose (HK) assay kit (Sigma-Aldrich, St. Louis, MO) and Synergy HT Multi-Mode microplate reader (BioTek, Wonooski, VT). Insulin concentration was determined in plasma in duplicate using a Mercodia Ovine Insulin ELISA kit and Mercodia Insulin Animal Controls (Mercodia USA, Winston Salem, NC) according to the manufacturer using a Synergy HT Multi-Mode microplate reader. Nonesterified fatty acids (NEFA) concentration was determined in serum using a MaxDiscovery™ Non-esterified fatty acids (NEFA) Assay Kit (Bioo Scientific, Austin, TX) according to the manufacturer's directions using a Synergy HT Multi-Mode microplate reader.

Instrumental color. At 24 h postmortem, color measurements were determined of the exposed *Longissimus* muscle (LM) and SQ at the 12th rib. The CIE Lab System was implemented and color measurements were recorded for L* (measures darkness to lightness; lower L* value indicates a darker color), a* (measures redness; higher a* value indicates a redder color), and b* (measures yellowness; higher b* value indicates more yellow color) using a Minolta chromameter (CR-310, Minolta Inc., Osaka, Japan) with a 50-mm-diameter measurement area using a D65 illuminant. The instrument was calibrated using the ceramic disk provided by the manufacturer. Color values were recorded at three locations of each tissue to obtain a representative reading.

Muscle pH and temperature. Muscle pH and temperature were measured in LM and ST muscles at 30 min, 1, 2, 3, 4, 5, 6, 12, and 24 h postmortem. Muscle pH was

measured by electrode insertion using a Eutech waterproof pH Spear (Cole-Parmer Instrument Company, Vernon Hills, IL) pH meter. Temperature was recorded by probe insertion at the same position using a Super-Fast Waterproof Pocket Thermometer (ThermoWorks Inc., American Fork, UT).

Muscle and liver glycogen/glucose and lactate. At the same postmortem time and in the same place of pH and temperature measurement, samples of LM and ST were removed, trimmed of all external fat and epimysial connective tissue, chopped, flash frozen in liquid nitrogen and stored at -80°C. Liver samples were taken 30 min postmortem, chopped, flash frozen in liquid nitrogen and stored at -80°C. These samples were used for glycogen/glucose determination by two different techniques: acid hydrolysis or enzymatic hydrolysis.

Acid hydrolysis technique. Duplicate 1.5 g samples were homogenized in 20 mL 2 *N* hydrochloric acid, incubated 20 min at 4°C, centrifuged at 2630 *x g* for 15 min at 4°C, filtered through Whatman paper and incubated 2 h at 90°C. Samples were cooled in an ice bath and an aliquot (10 mL) was neutralized with 2 *N* sodium hydroxide. The resulting hydrolyzed glycogen as glucose and glucose-6-phosphate were measured using a Glucose (HK) assay kit (Sigma-Aldrich) that is based on coupled enzyme reactions catalyzed by hexokinase and glucose-6-phosphate dehydrogenase. The increase in absorbance at 340 nm was measured in a 96-well plate using Synergy HT Multi-Mode microplate reader.

Enzymatic hydrolysis technique. Duplicate 0.5 g samples were homogenized in 15 mL 0.03 *M* hydrochloric acid. Five hundred μL of homogenate were taken and the pH

was adjusted to 4.8 with 1 *M* potassium bicarbonate. An aliquot of 100 μL was kept in ice for free glucose determination. The rest of the homogenate was incubated with 25 μL of alpha amylase during 10 min at 100°C. Samples were cooled and incubated with 30 μL of amyloglucosidase for 2 h at 40°C. The resulting hydrolyzed glycogen as total glucose was measured using a Liquid glucose (oxidase) reagent kit (Pointe Scientific), which is based on coupled enzyme reactions catalyzed by glucose oxidase and peroxidase. The increase in absorbance at 500 nm was measured in a 96-well using a Synergy HT Multi-Mode microplate reader. Free glucose was measured using the same procedure and the concentration of glycogen was calculated by subtracting free glucose from total glucose.

Acid and enzymatic hydrolysis techniques were compared in LM at 30 min, 2, 4 and 6 h postmortem. Glycogen/glucose concentration was lower ($P < 0.0001$) with acid hydrolysis compared to enzymatic hydrolysis regardless the water supplementation treatment (Figure 3.1). There was a significant interaction between postmortem time and technique ($P = 0.027$); the acid technique underestimated the amount of total glucose by 27, 31, 36 and 33 % at 30 min, 2, 4 and 6 h, respectively. Therefore, ST and liver glycogen and glucose were determined by the enzymatic technique. Lactate concentrations in LM and ST samples were determined according to Volpi-Lagreca and Duckett (2016).

Water holding capacity. Water holding capacity was measured in LM samples aged at 4°C for 1, 3, or 6 d using a centrifugation procedure that measures expressible moisture. Expressible moisture was measured in triplicate on LM samples according to the method of Allison et al. (2002) with some modifications. Three pieces of LM of

approximately 5 g were wrapped in Whatman filter paper #2 110 mm diameter (GE Healthcare UK Ltd., Buckinghamshire, UK), placed in a 50-ml centrifuge tube to be centrifuged at 8,400 x g for 30 min at 4 °C, and reweighed. Expressible moisture was calculated as the percentage of weight lost from the original weight of the sample before centrifugation.

Longissimus muscle proximate composition. One LM chop (2.54 cm thick) from the 12th rib trimmed of all external fat and epimysial connective tissue was vacuum packaged for proximate composition and fatty acid profile analysis. Samples were chopped (Blixer®3 Series D, Robot Coupe Inc., Ridgeland, MS) to reduce particle size. Duplicate samples of 2.5 g were removed for moisture content determination by weight loss by drying at 95°C for 24 h. The remaining samples were frozen at -20°C, lyophilized (VirTis, SP Scientific, Warminster, PA), ground (Blixer®3 Series D), and stored at -20°C for further analyses. Total lipids were extracted from freeze dried samples using an Ankom XT15 extractor (Ankom Technologies, Macedon, NY) with hexane as the solvent. Nitrogen content was determined by the combustion method using a Leco FP-2000 N analyzer (Leco Corp., St. Joseph, MI). Crude protein was calculated multiplying nitrogen content by 6.25. Total ash content was determined as residual weight after 600°C for 8 h (AOAC, 2000).

Fatty acid profile. Freeze dried samples of LM, SQ and liver were transmethylated according to Park and Goins (1994). Fatty acid methyl esters (FAME) were analyzed using an Agilent 6850 (Agilent, San Fernando, CA) gas chromatograph equipped with an Agilent 7673A (Hewlett-Packard, San Fernando, CA) automatic

sampler. Separation of FAME was accomplished using a Supelco 100-m SP2560 (Sigma-Aldrich, St. Louis, MO) capillary column (0.25 mm i.d. and 0.20 μm film thickness) using hydrogen as the carrier gas at a flow rate of 1 mL/min. Column oven temperature was programmed to increase from 150 to 174°C at a rate of 2°C/min, from 174 to 178°C at 0.2°C/min, from 178 to 225°C at 2°C/min, and then held at 225°C for 8.5 min. The injector and detector were maintained at 250°C. Sample injection volume was 1 μl. Samples were run twice with a split ratio of 100:1 for *trans* C18:1 and long chain fatty acids and again at split ratio of 10:1 for CLA and omega-3 fatty acids. Individual fatty acids were identified by comparing relative retention times with fatty acid standards (Sigma-Aldrich; Matreya, Pleasant Gap, PA). Fatty acids were quantified by incorporating methyl tricosanoic (C23:0) acid as an internal standard into each sample after methylation; values are expressed as g/100 g of total fatty acids.

Gene expression. Total cellular RNA (tcRNA) was extracted from liver tissue (Trizol, Invitrogen, Carlsbad, CA; PureYield, Promega, Madison, WI), converted to cDNA (Superscript III; Invitrogen), and analyzed by qPCR (QuantiTect SYBR Green PCR kit, Qiagen, Valencia, CA) for relative gene expression according to Duckett et al. (2009). Primers for genes of interest were designed on or spanned exon boundaries, when possible, using Primer3 software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/, accessed 23 March 2016). Primer sequences for peroxisome proliferator-activated receptor-α (PPARα), carnitine palmitoyltransferase 1 a (CPT1a), fatty acid binding protein 4 (FABP4), facilitated glucose transporter member 4 (GLUT4), acetyl-CoA carboxylase (ACC), fatty acid synthase (FASN), stearoyl-CoA

desaturase-1 (SCD1), fatty acid elongase-5 (ELOVL5), fatty acid elongase-6 (ELOVL6), and glyceraldehyde 3-phosphaste dehydrogenase (GAPDH) have been reported previously (Duckett et al., 2014). Primer sequences for other genes of interest, facilitated glucose transporter member 2 (GLUT2), glucose-6-phosphatase (G6PC), glycogen phosphorylase (PYGL), glycogen debranching enzyme (GDE), glycogen synthase 2 (GYS2), glycogen branching enzyme (GBE1), glutamic-pyruvate transaminase (GPT), and phosphoenol pyruvate carboxykinase (PCK1) are shown in Table 3.2. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene for data normalization. Normalized CT values ($\Delta CT = CT,gene - CT,GAPDH$) were calculated for each sample and subjected to ANOVA, as described below. The fold changes in gene expression for GLY or FRU vs. CON were calculated by using the $2^{-\Delta\Delta CT}$ method, according to Livak and Schmittgen (2001) and reported as fold changes from CON.

Statistical Analyses. Data were analyzed as a randomized complete block design using mixed procedure of SAS (SAS Inst. Inc., Cary, NC). During the grazing period 2 lambs died of a congenital heart defect, therefore data were analyzed for 16 lambs. For water intake, pen was used as the experimental unit (3 per treatment), and data were analyzed based in the following model: $Y_{ijk} = \mu + \beta_i + \tau_j + \beta\tau_{ij} + \delta_k + \tau\delta_{jk} + \varepsilon_{ijk}$, where Y_{ijk} is the observed value, μ is the overall mean, β_i is the block effect (i = 1 to 3), τ_j is the treatment effect (j = 1 to 3), $\beta\tau_{ij}$ is the random interaction between block and treatment, δ_k is the day effect (k = 1 to 30), $\tau\delta_{jk}$ is the interaction between treatment and day, and ε_{ijk} is the experimental error. For animal performance, carcass traits, LM proximate

composition, FA profile, liver composition, and gene expression, animal was used as experimental unit, and data were analyzed based in the following model: $Y_{ijk} = \mu + \beta_i + \tau_j + \beta\tau_{ij} + \alpha_k (\beta_i \tau_j)$, where Y_{ijk} is the observed value, μ is the overall mean, β_i is the block effect (i = 1 to 3), τ_j is the treatment effect (j = 1 to 3), $\beta\tau_{ij}$ is the random interaction between block and treatment, and $\alpha_k (\beta_i \tau_j)$ is the random animal effect within block and treatment (k = 1 to 16). For water holding capacity, the model also included day and their interaction. For muscle glycogen/glucose and lactate concentrations, animal was used as experimental unit, and data were analyzed based in the following model: $Y_{ijk} = \mu + \beta_i + \tau_j + \beta\tau_{ij} + \alpha_k (\beta_i \tau_j) + \delta_m + \tau\delta_{jm} + \delta_m (\alpha_k \tau_j) + \varepsilon_{ijk}$, where Y_{ijk} is the observed value, μ is the overall mean, β_i is the block effect (i = 1 to 3), τ_j is the treatment effect (j = 1 to 3), $\beta\tau_{ij}$ is the random interaction between block and treatment, and $\alpha_k (\beta_i \tau_j)$ is the random animal effect within block and treatment (k = 1 to 16), δ_m is the postmortem time effect (m = 1 to 9), $\tau\delta_{jm}$ is the interaction between treatment and postmortem time, $\delta_m (\alpha_k \tau_j)$ is the random postmortem time effect within animal and treatment, and ε_{ijkmn} is the experimental error. For pH the model also muscle and their interaction, whereas for glycogen hydrolysis techniques comparison the model also included technique and their interaction. Least square means were generated and separated by Fisher's protected LSD. Significance was determined at $P < 0.05$. Differences of $P > 0.05$ to $P < 0.10$ are discussed as trends. For gene expression data, when the overall F-test for the supplementation treatment effect was significant ($P < 0.05$), preplanned, a priori contrasts were used to test differences among FRU vs. CON and GLY vs. CON.

RESULTS AND DISCUSSION

Animal performance and water intake are shown in Tables 3.3 and 3.4, respectively. Average daily gain during the 28-d grazing period was higher ($P = 0.018$) for lambs supplemented with GLY compared to CON or FRU, which did not differ ($P > 0.05$). Fat thickness measured by ultrasound method at the beginning and at the end of the grazing period did not differ among treatments ($P > 0.05$), thus the rate of SQ accretion was similar ($P > 0.05$) between treatments. During this 28-d period, water intake was not altered ($P > 0.05$) by treatment. The intake of glycerol averaged 0.397 kg per lamb daily for GLY and was similar ($P > 0.05$) to intake of fructose (0.342 kg per lamb daily) for FRU. Others have reported no differences in water intake of lactating dairy cows supplemented with 2% glycerin (Osborne et al., 2009) or 2% glucose (Osborne et al., 2002) in drinking water compared to control.

During the fasting period (last 2 d prior to slaughter), CON and FRU animals lost similar BW (1.51 kg/d, $P > 0.05$) and it was 3.85 times higher than the weight loss of GLY animals (Table 3.3). Chelikani et al. (2004) reported a decrease in body weight of Holstein cows and heifers by 48 h of fasting. Beverages containing glycerol are utilized in endurance exercise athletes to hyperhydrate body tissues and increase fluid retention (van Rosendal et al., 2010). They find that glycerol is osmotically active and evenly dispersed throughout the body, which helps to balance fluid retention during heavy exercise. Thus, the intake of glycerol during the fasting period may have mitigated BW loss of GLY lambs compared to CON and FRU due to hyperhydration and/or supply of

substrates for gluconeogenesis to prevent mobilization from tissue storage depots. During the fasting period, water intake tended (*P* = 0.08) to be higher for GLY compared to FRU, resulting in a higher intake of substrates (0.421 kg/lamb/d for GLY vs. 0.271 kg/lamb/d for FRU). Average intake of glycerol during the fasting period was 55% higher than the intake of fructose, whereas CON animals did not consume any substrates, only water, during this 2-d period.

Plasma glucose was higher (*P* < 0.05) for GLY compared to CON or FRU, which were similar (*P* > 0.05; Figure 3.2). Gardner et al. (2001) reported an increase in plasma glucose concentration when sheep fed low energy diets were offered water containing 3.5% glycerol and 1.5% propylene glycol over 24 h period. Others (Chelikani et al. 2004), reported a rapid decline in plasma glucose concentration of cows fasted for 48 h relative to cows fed ad libitum. Plasma insulin level was not affected by treatment (*P* > 0.05). Serum NEFA concentration was higher (*P* < 0.05) for CON and FRU compared to GLY, which would indicate a lower lipid mobilization in GLY animals during fasting. Others (Chelikani et al., 2004; McVeigh and Tarrant, 1982), reported an increase in NEFA in heifers in response to fasting.

Hot carcass weight was 29% greater (*P* = 0.036) for GLY than FRU (Table 3.5). Fat thickness tended (*P* = 0.08) to be higher for GLY than FRU. Dressing percentage and ribeye area were not affected by treatment (*P* > 0.05). These results indicate that differences in weight loss at fasting were not related to ruminal fill; GLY animals gained more weight during the grazing period and lost less weight during the fasting period, resulting in heavier carcasses. These results suggest that hyperhydration and fluid

retention with glycerol (van Rosendal et al., 2010) may be a reason for the increased BW gain and heavier carcass weight.

Longissimus muscle color (L*, a* and b*) and SQ color (L*, a* and b*) were not affected ($P > 0.05$) by treatment (Table 3.5). Changes in lean color have been attributed to higher ultimate pH, which is associated with lower lightness, greater redness, and a darker meat color (Guignot et al., 1994; Wulf et al., 1997; Abril et al., 2001). In this study, ultimate pH in the LM was in the normal range and averaged 5.68 across all treatments (Figure 3.3). *Longissimus* proximate composition did not differ ($P > 0.05$) among treatments.

Muscle pH was not affected by treatment ($P > 0.05$), but it was affected by postmortem time ($P < 0.0001$) and muscle ($P < 0.0001$) (Figure 3.3). There was a significant interaction between postmortem time and muscle ($P < 0.0001$). For LM, the pH decreased ($P < 0.05$) as postmortem time increased to 24 h, whereas for ST the pH decreased the first 12 h postmortem but did not decrease further from 12 to 24 h postmortem ($P = 0.42$), indicating that ST reached the ultimate pH at 12 h postmortem. LM showed a higher pH than ST during the first 12 h at each postmortem time ($P < 0.05$), and the pH values were similar ($P > 0.05$) for both muscles at 24 h. It has been shown that ST had lower frequency of type I fibers and higher frequency of type IIB fibers than LM (Briand et al., 1981; Vestergaard et al., 2000). Type I fibers are characterized by an oxidative metabolism, whereas type IIB fibers have a glycolytic metabolism (Lefaucheur and Gerrard, 2000). Ryu and Kim (2005) reported a negative relationship between pH at 45 min postmortem and the number of type IIB (glycolytic)

fibers and a positive relationship with the number of type I (oxidative) fibers. The faster glycolytic rate of type IIB fibers which are reported to be more abundant in ST (Briand et al., 1981; Vestergaard et al., 2000), may explain why ST reached the ultimate pH before than LM.

Glycogen/glucose and lactate concentrations are presented in Figure 3.4 for LM and in Figure 3.5 for ST. In both muscles, glycogen/glucose concentration decreased as postmortem time increased ($P < 0.0001$). Water supplementation treatment did not affect ($P > 0.05$) the average concentration of glycogen/glucose neither in LM nor in ST. However, there was a significant interaction between treatment and postmortem time ($P = 0.003$) for LM. The concentration of glycogen/glucose in FRU animals was higher than CON at 1 h postmortem and tended ($P < 0.10$) to be higher than CON at 6, 12 and 24 h postmortem. Animals receiving GLY had higher ($P < 0.05$) concentrations of glycogen/glucose at 2 and 3 h postmortem and tended ($P < 0.10$) to have higher concentration than CON at 30 min and 24 h postmortem. Despite these differences, the concentration of muscle glycogen at early postmortem times was high for all animals, indicating that muscle glycogen was not considerably depleted during the fasting period. A slow depletion of muscle glycogen appears to occur during starvation in rested animals. Glycogen depletion in living muscle may be triggered by either increased catecholamine (epinephrine) levels or muscle contraction, or by both mechanisms acting in concert (Tarrant, 1989). The rate of breakdown of muscle glycogen in epinephrine-treated or socially-regrouped cattle has been shown to be around 185 times greater than the rate of depletion in cattle at rest under fasting conditions (Tarrant, 1989). The stress

that the animals experimented in our study was low, they were not socially regrouped during the fasting period and the transport from the farm to the meat laboratory for slaughter was short. A fasting period of 2 d with low stress was not detrimental in glycogen concentrations previous to slaughter. Similarly, 2 or 4 d of feed withdrawal had no effect on muscle glycogen concentration in sheep previously maintained in pasture and slaughtered under low stress conditions (Daly et al., 2006). Apparently, lipid mobilization helps the animals to spare muscle glycogen reserves during fasting (McVeigh and Tarrant, 1982). In our study, CON and FRU animals showed increased serum NEFA compared to GLY, which indicates that lipid mobilization may help this animals to maintain muscle glycogen levels whereas in GLY animals muscle glycogen depletion may have been mitigated by the supply of glycerol as a gluconeogenic substrate, since the mobilization of lipids from fat depots in this animals was low as indicated by NEFA levels.

Longissimus and ST lactate concentrations were not affected ($P > 0.05$) by treatment. As postmortem time increase ($P < 0.0001$) lactate concentration increased. Lactate accumulates from glycogen as an end product of anaerobic glycolysis postmortem; in absence of oxygen, pyruvate is converted to lactate by the enzyme lactate dehydrogenase. Lactate accumulation is responsible for postmortem pH decline. Lowe et al. (2004) reported that concentrations of glycogen in the muscle measured 45 min postmortem below 25 mmol/kg of fresh tissue resulted in a high ultimate pH of the meat (above 5.9). In this study, residual glycogen/glucose concentrations at 30 min postmortem were more than 2 times that value in both muscles, thus the resulting

ultimate pH was normal (5.68 for LM and 5.73 for ST). These results may indicate that the glycogen content in the muscles at the moment of slaughter was sufficient for fueling lactic acid production resulting in a normal ultimate pH in both LM and ST muscles.

Longissimus and ST comparisons at 30 min, 2, 4, and 6 h postmortem for glycogen/glucose concentrations, determined in both muscles by enzymatic hydrolysis, and lactate concentrations are presented in Figure 3.6. ST showed lower ($P < 0.05$) concentrations of glycogen/glucose at each postmortem time. There was a significant interaction between muscle and postmortem time ($P = 0.008$); the magnitude of the difference between LM and ST was lower at 30 min postmortem and increased at 2, 4, and 6 h. Additionally, lactate concentration was higher ($P < 0.05$) for ST at each postmortem time. According to Choe et al. (2008), muscles with lower glycogen and higher lactate content indicate a faster glycolytic rate at the early postmortem period than muscles with higher glycogen and lower lactate content. The results from our study support this finding.

Water holding capacity of LM, determined as expressible moisture (Figure 3.7), was not affected by treatment ($P > 0.05$) in agreement with previous results reported by our lab for steers supplemented with glycerol or high fructose corn syrup via drinking water (Volpi-Lagreca and Duckett, 2016). The ability of meat to retain water is influenced by the rate and extent of pH decline as well as the proteolysis of muscle fibers postmortem (Huff-Lonergan and Lonergan, 2005) due its influence on protein to protein interactions (Hamm, 1975). In our study, LM pH decline did not differ ($P > 0.05$) between treatments (Figure 3.3). Water holding capacity was affected by postmortem

aging time (*P* = 0.0003); it decreased (or expressible water increased) from 1 to 3 d postmortem without further changes after 3 d postmortem. Kristensen and Purslow (2001) observed a decrease in WHC the first 2 d postmortem followed by an increase in WHC after 7 d of storage. The authors suggested that degradation of cytoskeleton during aging would increase WHC of meat by removing inter-myofibrillar connections, reducing the linkage between lateral shrinkage of myofibrils and shrinkage of entire muscle fibers that squeezes out water.

Liver total lipid content (Figure 3.8) was higher ($P > 0.05$) for CON than GLY with FRU being intermediate. During fasting, animals mobilize lipids from fat depots to the liver, where the lipids are accumulated as triglycerides and redistributed to tissues by VLDL proteins (Grummer, 1993). Carr et al. (1973) reported an increase in liver total lipids by fasting; furthermore, the percentages of total lipids in liver increased with each additional day of fast. In this study, it would indicate that supplementation of gluconeogenic substrates, both FRU and GLY, reduced lipid mobilization with GLY having a greater effect. This is supported by the lower level of serum NEFA found in GLY animals at slaughter.

Liver glycogen content was higher ($P < 0.05$) for GLY compared to CON or FRU, which were similar ($P > 0.05$) between them (Figure 3.8), whereas liver free glucose was higher for GLY, intermediate for FRU and lower for CON animals ($P < 0.05$, Figure 3.8). Liver glycogen is an important source of energy reserve. It has been shown that liver glycogen content decrease in fasted cattle (Carr et al., 1973). In this

study, glycerol supplementation appeared to avoid the depletion of liver glycogen reserves that would normally occur during fastening.

Fatty acid profiles of the LM, SQ, and liver are shown in Tables 3.6, 3.7 and 3.8, respectively. Supplementation with GLY increased ($P < 0.05$) total odd-chain fatty acid concentrations in LM and liver. In the LM, margaric (C17:0) acid was higher ($P = 0.036$) for GLY than FRU or CON; whereas pentadecyclic (C15:0) acid increased ($P < 0.0001$) in liver and tended to increase ($P = 0.059$) in SQ with GLY. Odd-chain fatty acids are produced in ruminant adipose tissues when propionate (3C) is used in fatty acid synthesis instead of acetate (2C) which generates C15:0 or C17:0 fatty acid (Horning et al., 1961). Increased odd-chain fatty acid accumulation in the tissues would suggest a greater proportion of propionate synthesis by rumen microbes. Wang et al. (2009) observed a linear decrease in ruminal pH and a linear increase in total VFA concentration in steers with increasing dietary glycerol supplementation. Additionally, the ratio of acetate to propionate decreased as glycerol supplementation increased due to the increased in propionate production. Glycerol that enters the rumen is rapidly fermented to volatile fatty acids. Ramos and Kerley (2012) reported linear increases in propionate and linear decreases in acetate production when glycerol was added at increasing levels in continuous culture systems. As indicated by the bypass studies (Woodford et al., 1984; Garza and Owens, 1989), not all glycerol in drinking water would bypass ruminal fermentation and therefore the glycerol that enters the rumen would increase propionate production in the rumen. These changes in rumen fermentation would supply more

propionate and resulted in increased deposition of odd-chain fatty acids observed in all tissues measured.

Supplementation with FRU and GLY reduced stearic (C18:0) acid concentrations in LM ($P = 0.064$) and SQ ($P = 0.018$) compared to CON. In SQ, oleic (C18:1 cis-9) acid concentration tended to be greater ($P = 0.066$) for FRU and GLY compared to CON. Concentrations of stearic and oleic acids in the tissues are related to the extent of biohydrogenation in the rumen of dietary unsaturated fatty acids and desaturation of stearic acid by stearoyl-CoA desaturase (SCD1) in the tissues. These results would suggest that ruminal biohydrogenation may be reduced with GLY supplementation and/or SCD1 may be upregulated at the adipose tissue level. In contrast, liver concentrations of stearic (C18:0) acid tended to be higher (P = 0.085) for GLY than CON. Docosahexanoic (C22:6; DHA) acid concentration tended to be increased ($P = 0.057$) in LM with GLY supplementation compared to CON. In the liver, concentrations of all long-chain n-3 fatty acid (EPA, DPA, DHA) were elevated ($P < 0.05$) with FRU and GLY supplementation. Linolenic (C18:3 n-3) acid concentration was reduced ($P = 0.031$) in both FRU and GLY supplemented liver samples. Overall n-3 polyunsaturated fatty acid concentration was increased ($P = 0.022$) by 37% for FRU and GLY compared to CON. α-Linolenic acid (C18:3 n-3; ALA) is an essential fatty acid that must be supplied in the diet as it cannot be synthesized by mammals (Nakamura and Nara, 2003). Once ALA is supplied in the diet, it can be elongated and desaturated to produce EPA, DPA, and DHA (Nakamura and Nara, 2003); however, estimates are that the efficiency of conversion to long-chain n-3 fatty acids is low in humans (21% ALA to EPA for

women; 8% ALA to EPA for men; Desci and Kennedy, 2011) and in pigs (25% ALA to EPA; Smink et al., 2013). Based on these results, we would propose that supplementation of GLY and FRU via drinking water reduced ruminal biohydrogenation of linolenic acid and thereby supplying it to the tissues where further conversion to EPA, DPA, and DHA was stimulated with FRU and GLY supplements.

The expression of genes related to lipid metabolism in liver is shown in Figure 3.9. The mRNA expression of CPT1a tended ($P < 0.10$) to be lower with glycerol supplementation relative to CON. CPT1 is a regulatory enzyme in the mitochondria that transfers fatty acids from the cytosol to the mitochondria for β-oxidation (Berlanga et al., 2014). However, the expression of PPARα, enzyme that upregulates fatty acid oxidation systems (Berlanga et al., 2014), did not differ ($P > 0.05$) between treatments. The greater CPT1 gene expression in CON animals may be due to a greater utilization of fatty acids in the liver compared to GLY, in which the accumulation of lipids in the liver after fasting was lower. FABP4 expression was higher ($P < 0.05$) in CON than in GLY; FABP4 is involved in binding of free fatty acids for transport. Westerbacka et al. (2007) reported an increase in the expression of FABP4 in human liver with higher fat content. The higher expression in CON animals compared to GLY and the higher total lipid content found in CON are in agreement with this finding. The mRNA expression of key lipogenic genes such as ACC, FASN and SCD1 was upregulated ($P < 0.05$) in GLY compared to CON, whereas FASN mRNA expression tended ($P < 0.10$) to be upregulated with FRU relative to CON. High fructose diets induced higher mRNA

expression of ACC, FASN, and SCD1 in mouse liver (Miyazaki et al., 2004) and ACC and FASN in rat (Janevski et al., 2012).

The relative expressions of genes related to glucose metabolism in liver are shown in Figure 3.10. Glycerol supplementation upregulated ($P < 0.05$) the mRNA expression of GLUT2 and GLUT4 relative to CON, whereas FRU upregulated ($P < 0.05$) the expression of GLUT4 and tended ($P < 0.10$) to upregulate the expression of GLUT2. These enzymes are membrane proteins that regulates transport of glucose; GLUT2 mediates the bidirectional transfer of glucose across the plasma membrane of hepatocytes whereas GLUT4 is an insulin-regulated facilitative glucose transporter. The increase in mRNA expression of these genes may indicate a greater supply of gluconeogenic substrates in the liver of supplemented animals. Asano et al. (1992) reported an increase in GLUT2 mRNA levels in isolated rat hepatocytes cultured with glucose or fructose.

The enzyme G6PC catalyzes the final step in the gluconeogenic and glycogenolytic pathways, and therefore plays a key role in maintaining blood glucose levels in the fasted state (Hutton and O'Brien, 2009), however, there were no differences ($P > 0.05$) in G6PC mRNA expression between treatments. Martin et al. (1973) reported that G6PC concentration in ovine liver was decreased by prolonged fasting. Probably a 2-d fasting period in our study was not long enough to find differences in G6PC mRNA expression.

Changes in OCFA would indicate an increase in rumen propionate production in supplemented animals. In the liver, propionate is converted to oxaloacetate, which can be metabolized by PCK1 to phosphoenolpyruvate and further to glucose. The abundance of

PCK1 in the cytosol regulates propionate entry into gluconeogenesis (Aschenbach et al., 2010). White et al. (2016) found an increase in PCK1 expression in the liver of cows fed glycerol compared to control, maybe as a consequence of increased propionate supply to the liver from diets containing glycerol. In our study, the mRNA expression of PCK1 tended ($P < 0.10$) to be upregulated with FRU relative to CON, but there were no differences ($P > 0.05$) between GLY and CON.

In fasted ruminants, amino acids are the main supply of carbon for glucose (Martin et al., 1973), whereas pyruvate formed by glycolysis in the muscle can undergo transamination to alanine and be used for gluconeogenesis in the liver (Bender and Mayes, 2012). The enzyme GPT plays a key role in the intermediary metabolism of glucose and amino acids in the liver, therefore, an increase in GPT mRNA in animals that did not receive any supply of gluconeogenic substrates during the fasting period (i.e. CON) would be expected. However, GPT mRNA did not differ ($P > 0.05$) between CON and supplementation treatments. Similarly, Martin et al. (1973) reported that GPT concentration was not altered in ovine liver by prolonged fasting.

The mRNA expression of genes associated with to glycogen synthesis was contradictory. The expression of GYS2 tended ($P < 0.10$) to be downregulated with GLY relative to control, however, GBE1 tended ($P < 0.10$) to be upregulated with GLY. On the other hand, there were no significant differences ($P > 0.05$) in mRNA expression of genes related to glycogen degradation such as PYGL and GDE. The enzymes glycogen synthase and glycogen phosphorylase are regulated in opposite directions by post-translational phosphorylation and dephosphorylation. Phosphorylation of glycogen

phosphorylase increases its activity, whereas phosphorylation of glycogen synthase reduces its activity.

Overall, 12% glycerol supplementation via drinking water increased ADG in grazing lambs, reduced BW shrink during the fasting period previous to slaughter, and increased HCW. Muscle glycogen contents were increased at early postmortem times with gluconeogenic substrates supplementation, however postmortem pH decline and ultimate pH were similar across treatments. The normal ultimate pH would indicate that the glycogen content in the muscles at the moment of slaughter was sufficient in all animals for fueling lactic acid production postmortem; increased lipid mobilization in CON and FRU animals may have spared muscle glycogen depletion during fastening whereas in GLY animals muscle glycogen depletion may have been mitigated by the supply of glycerol as a gluconeogenic substrate. In the liver, glycerol supplementation favored glycogen and free glucose content, and upregulated mRNA expression of lipogenic and glucose transport genes. We conclude that 12% glycerol supplementation via drinking water may favor the growth rate in grazing ruminants, reduce BW loss during fastening and increase glycogen contents in muscle and liver which will ensure a normal pH decline avoiding lipid mobilization that will occur during fastening.

Table 3.1. Chemical composition of alfalfa (*Medicago sativa*) grazed during the experiment.

Proximate analysis[a]	
Dry matter, %	21.2 ± 4.6
Crude Protein, % DM	20.7 ± 2.5
Ether extract, % DM	2.4 ± 0.2
NDF, % DM	43.7 ± 5.6
ADF, % DM	33.6 ± 4.6
Ash, % DM	6.8 ± 1.0

[a]Based on samples taken every 2 weeks

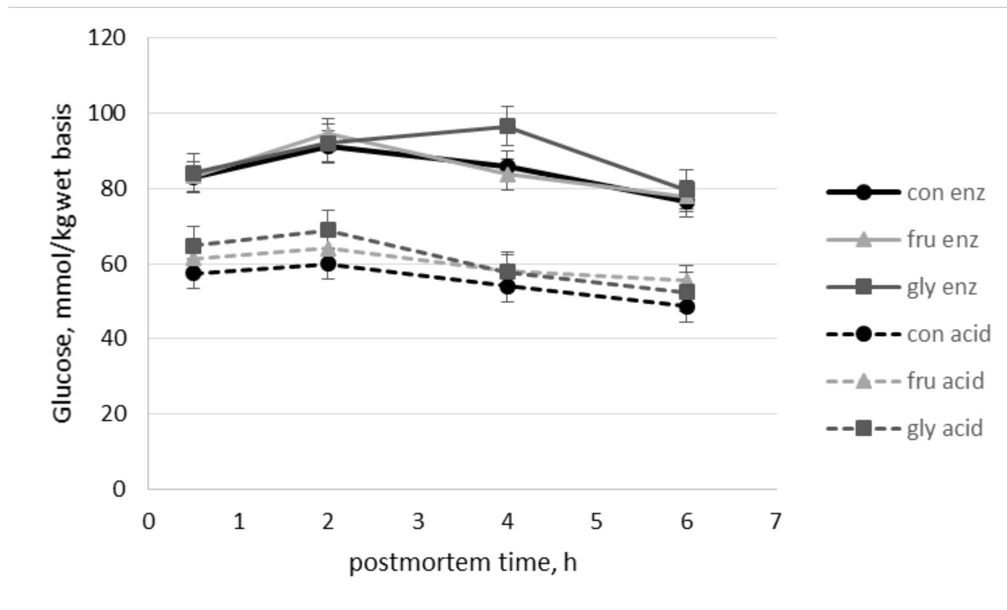


Figure 3.1. Glycogen/glucose concentration determined by acid hydrolysis (acid) or enzymatic hydrolysis (enz) techniques of *Longissimus* muscle at 30 min, 2, 4, and 6 h postmortem of lambs offered regular drinking water (CON) or supplemented with glycerol (GLY) or fructose (FRU) via drinking water. Glycogen/glucose concentration was not affected by treatment ($P = 0.68$) but was affected by postmortem time ($P < 0.0001$) and technique ($P < 0.0001$). There was a significant interaction postmortem time x technique ($P = 0.027$).

Table 3.2. Primer sequences (5' to 3') for quantitative real-time PCR.

Gene[1]	Forward	Reverse	Efficiency
GLUT2	GGTTCATGGTGGCTGAGTTT	TCCGCAATGTACTGGAAACA	1.95
G6PC	ATTGCGGTTGCTGAGACTTT	ATCCAATGGCGAAACTGAAC	1.99
PYGL	CCTGCACTTCACTCTGGTCA	ATAAACCCTCTTGGGGCACT	1.96
GDE	ATTGCGGTACATGGATGGAT	AATTCCAGCAACCAACGAAC	1.91
GYS2	CCGCCAGAGGATTATTCAAA	TGGGTTCCATGTGGAATTTT	1.99
GBE1	GGGCGAAATATGTGACTCGT	TTTTGGCTTCTTTGGTTTGG	1.93
GPT	GCCCATCTGAGCAGAGTCAT	CTCCGGACACAAGGATTCAT	1.96
PCK1	CCCCTGGAGATCAAGAATCA	CTGGTGCGTTGTATGGATTG	2.04

[1] GLUT2: Facilitated glucose transporter 2; G6PC: Glucose-6-phosphatase, catalytic subunit; PYGL: Glycogen phosphorylase (Liver); GDE: Glycogen debranching enzyme; GYS2: Glycogen synthase 2 (Liver); GBE1: Glycogen branching enzyme; GPT: Glutamic-pyruvate transaminase; PCK1: Phosphoenol pyruvate carboxykinase, cytosolic.

Table 3.3. Animal performance and ultrasound measurements of lambs offered regular drinking water (CON) or supplemented with glycerol (GLY) or fructose (FRU) via drinking water.

	CON	FRU	GLY	SEM	P-level
Grazing period (28 d)					
Initial body weight, kg	40.02	39.41	41.19	1.488	0.750
Final body weight, kg	43.26	41.60	46.79	1.530	0.223
Average daily gain, kg/d	0.116[a]	0.078[a]	0.200[b]	0.0128	**0.018**
Ultrasound measurements					
Initial fat thickness, mm	1.78	1.82	2.24	0.437	0.770
Final fat thickness, mm	3.30	3.60	3.99	0.548	0.727
SQ[1] accretion, mm/d	0.054	0.064	0.062	0.0090	0.731
Fasting period (2 d)					
Fasted body weight, kg	38.77	37.30	44.49	1.860	0.166
Weight loss, kg/d	1.680[a]	1.340[a]	0.392[b]	0.1762	**0.037**

[1]SQ: subcutaneous fat

[a,b]Means in a row followed by different superscripts differ ($P < 0.05$).

Table 3.4. Water intake of lambs offered regular drinking water (CON) or supplemented with glycerol (GLY) or fructose (FRU) via drinking water.

	CON	FRU	GLY	SEM	P-level
Grazing period (28 d)					
Water intake, L/lamb/d	4.46	2.91	3.02	1.107	0.564
Glycerol intake, kg/lamb/d	-	-	0.397		
Fructose intake, kg/lamb/d	-	0.342	-		
Fasting period (2 d)					
Water intake, L/lamb/d	2.73^{xy}	2.38^{x}	3.79^{y}	0.296	**0.080**
Glycerol intake, kg/lamb/d	-	-	0.421		
Fructose intake, kg/lamb/d	-	0.271	-		

^{x,y}Means in a row followed by different superscripts differ ($P < 0.10$).

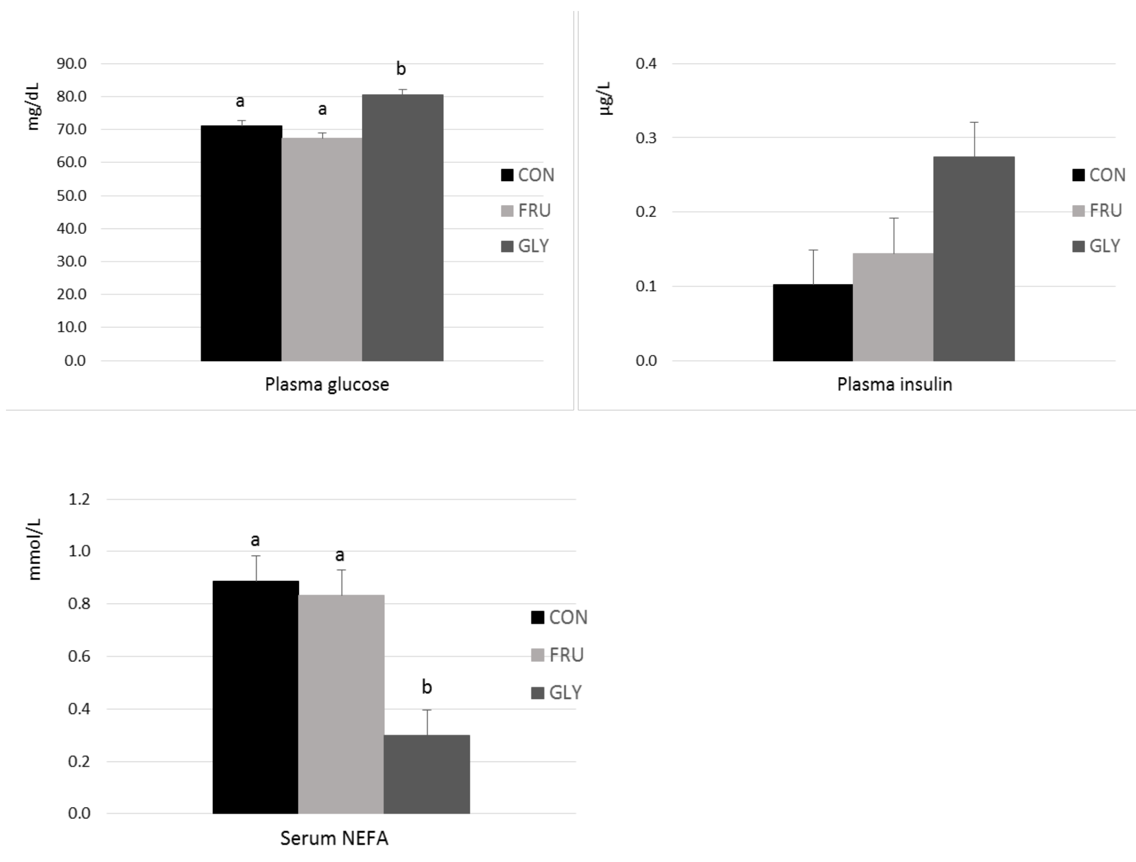


Figure 3.2. Blood glucose, insulin, and nonesterified fatty acids (NEFA) at slaughter of lambs offered regular drinking water (CON) or supplemented with glycerol (GLY) or fructose (FRU) via drinking water. Glucose and NEFA were affected by treatment ($P < 0.05$), whereas the effect of treatment on insulin was nonsignificant ($P = 0.18$). [a,b]Means with different letters are significantly different ($P < 0.05$).

Table 3.5. Carcass traits, instrumental color, and *Longissimus* muscle (LM) proximal composition of lambs offered regular drinking water (CON) or supplemented with glycerol (GLY) or fructose (FRU) via drinking water.

	CON	FRU	GLY	SEM	P-level
Hot carcass weight, kg	19.71^{ab}	17.59^{a}	22.67^{b}	0.685	**0.036**
Dressing percentage, %	50.54	47.75	50.79	1.817	0.482
Ribeye area, cm^2	14.19	14.30	15.05	0.681	0.707
Fat thickness, mm	3.39^{xy}	2.54^{x}	3.76^{y}	0.244	**0.080**
LM[1] L*	43.33	42.60	43.02	0.446	0.514
LM a*	24.54	23.99	23.89	0.446	0.578
LM b*	6.88	6.10	5.98	0.419	0.356
SQ[2] L*	83.12	80.30	78.26	1.317	0.176
SQ a*	8.49	9.36	11.62	0.809	0.170
SQ b*	11.96	11.26	12.07	0.981	0.815
Proximate composition of LM, g/100g					
Moisture	71.73	72.20	73.06	0.494	0.335
Total lipids	3.85	3.95	3.46	0.377	0.707
Crude protein	23.17	22.54	22.05	0.242	0.108
Ash	1.34	1.33	1.30	0.078	0.921

[1]LM: *Longissimus* muscle.

[2]SQ: subcutaneous fat.

[a,b]Means in a row followed by different superscripts differ ($P < 0.05$).

[x,y]Means in a row followed by different superscript differ ($P < 0.10$).

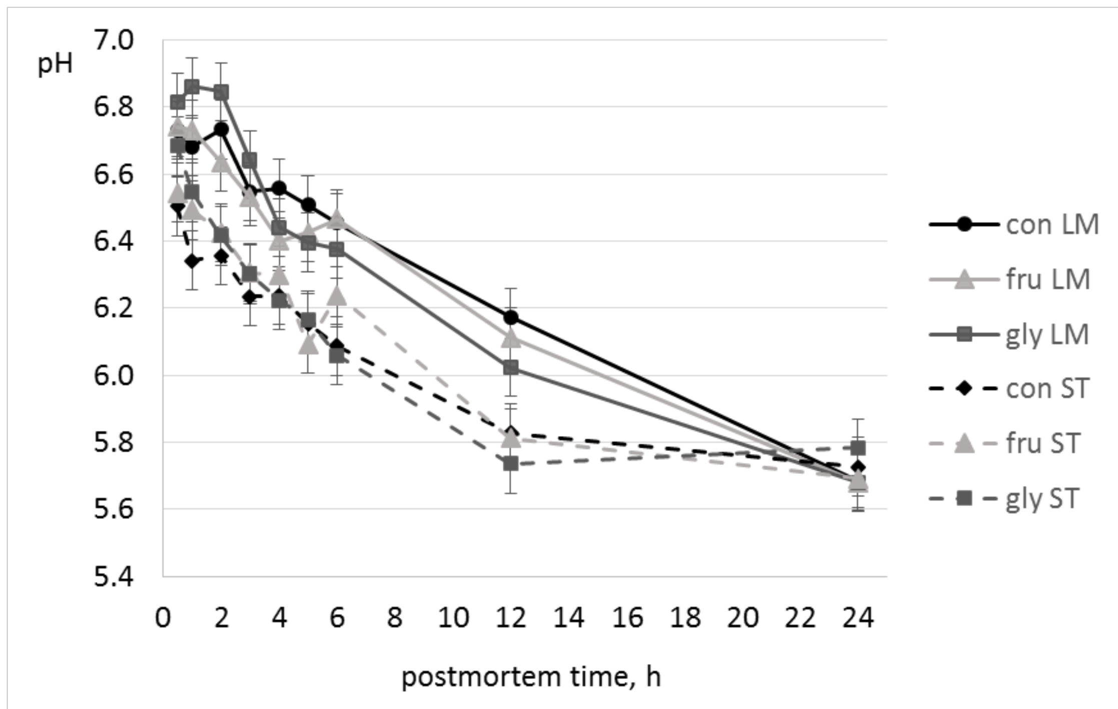


Figure 3.3. pH of *Longissimus* and *Semitendinosus* muscles at 30 min, 1, 2, 3, 4, 5, 6, 12, and 24 h postmortem of lambs offered regular drinking water (CON) or supplemented with glycerol (GLY) or fructose (FRU) via drinking water. pH was not affected by treatment ($P = 0.67$) but was affected by postmortem time ($P < 0.0001$) and muscle ($P < 0.0001$). There was a significant interaction postmortem time x muscle ($P < 0.0001$). Other 2- and 3-way interactions were not significant ($P > 0.05$).

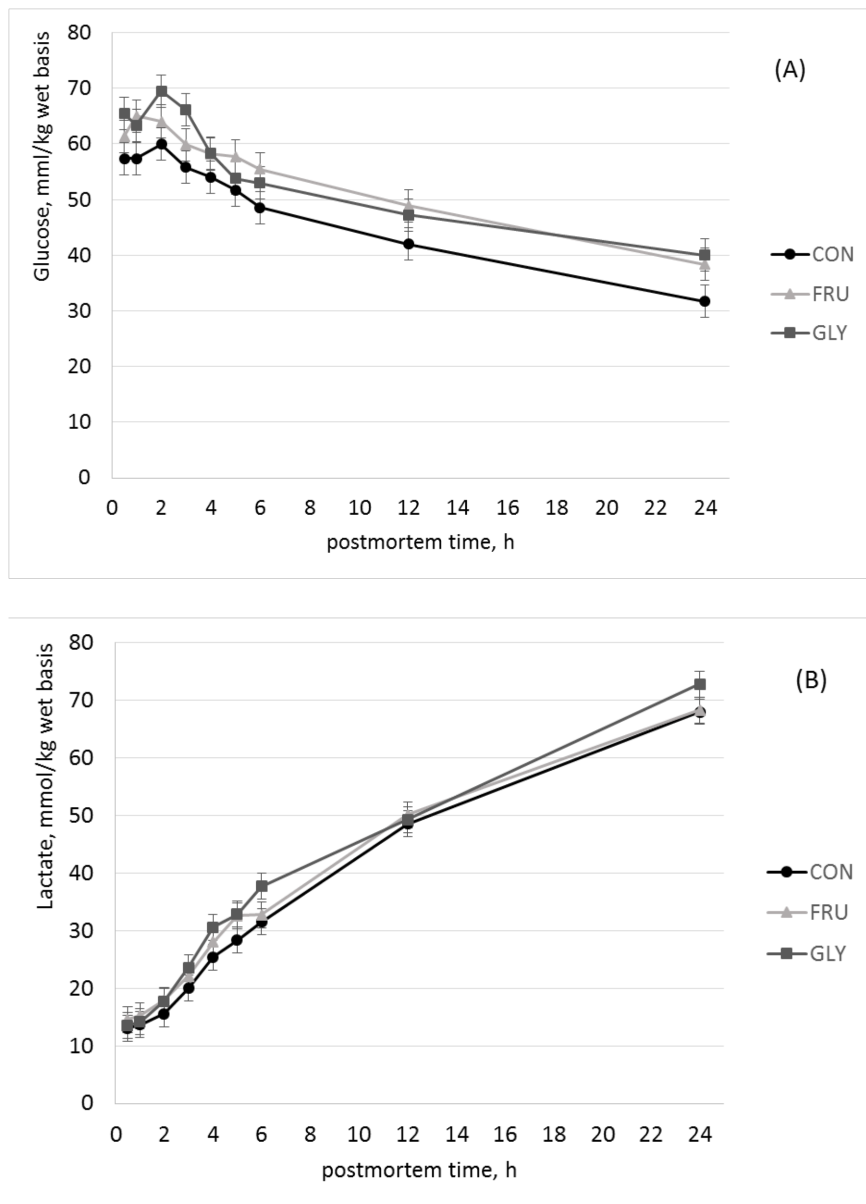


Figure 3.4. Glycogen/glucose concentration determined by acid hydrolysis (A) and lactate concentration (B) of *Longissimus* muscle at 30 min, 1, 2, 3, 4, 5, 6, 12, and 24 h postmortem of lambs offered regular drinking water (CON) or supplemented with glycerol (GLY) or fructose (FRU) via drinking water. Glycogen/glucose and lactate concentrations were not affected by treatment ($P > 0.05$) but were affected by postmortem time ($P < 0.0001$). There was an interaction treatment x postmortem time ($P < 0.05$) for glycogen/glucose concentration.

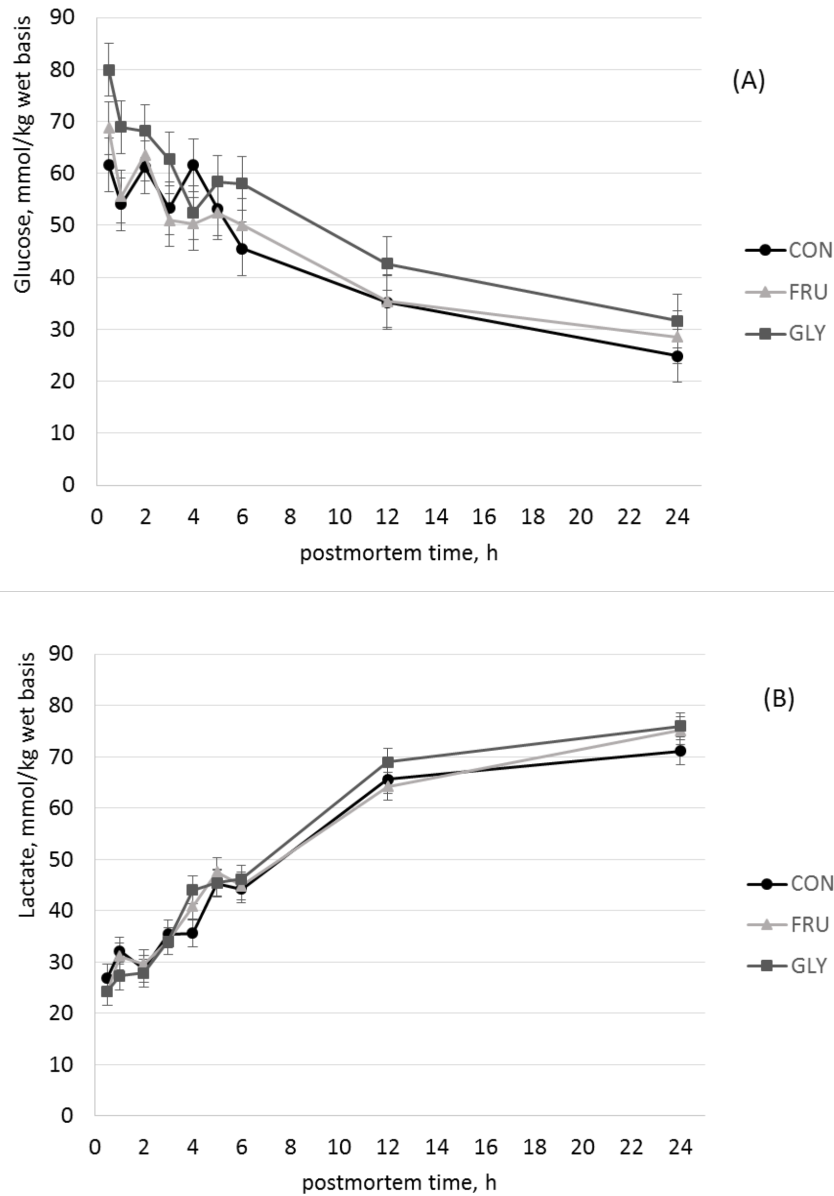
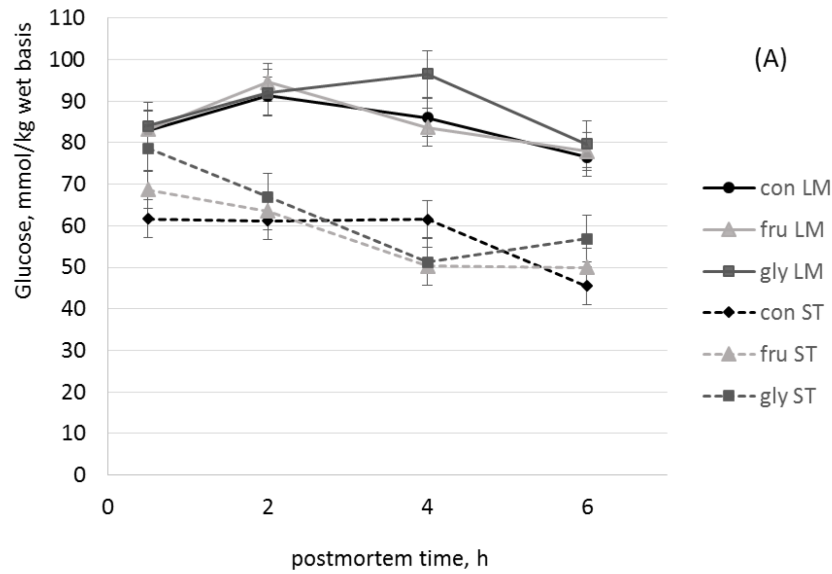


Figure 3.5. Glycogen/glucose concentration determined by enzymatic hydrolysis (A) and lactate concentration (B) of ST at 30 min, 1, 2, 3, 4, 5, 6, 12, and 24 h postmortem of lambs offered regular drinking water (CON) or supplemented with glycerol (GLY) or fructose (FRU) via drinking water. Glycogen/glucose and lactate concentrations were not affected by treatment ($P > 0.05$) but were affected by postmortem time ($P < 0.0001$). The interaction treatment x postmortem time was nonsignificant ($P > 0.05$).



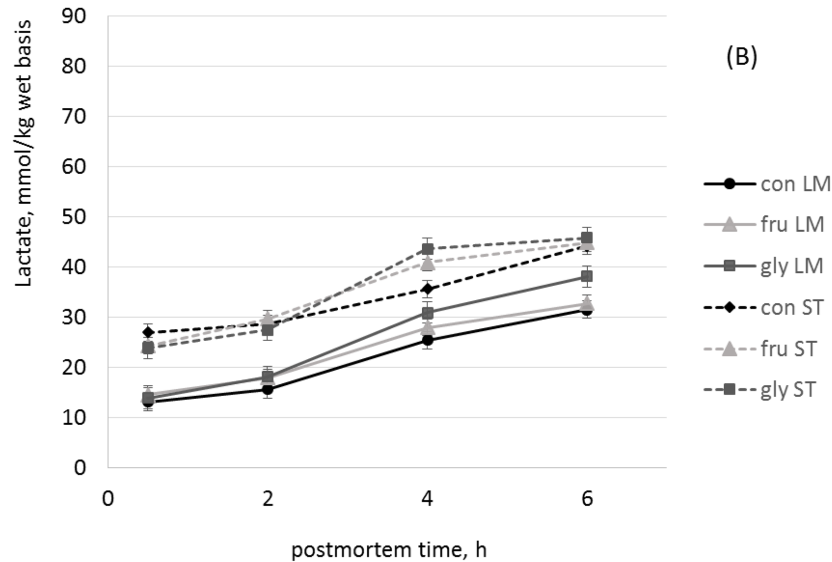


Figure 3.6. Glycogen/glucose (A) and lactate (B) concentrations of *Longissimus* and *Semitendinosus* muscles at 30 min, 2, 4, and 6 h postmortem of lambs offered regular drinking water (CON) or supplemented with glycerol (GLY) or fructose (FRU) via drinking water. Glycogen/glucose was determined by enzymatic hydrolysis in both muscles. Glycogen/glucose and lactate concentrations were not affected by treatment ($P > 0.05$) but were affected by postmortem time ($P < 0.0001$) and muscle ($P < 0.0001$). There was a significant interaction postmortem time x muscle ($P = 0.008$) for glycogen/glucose.

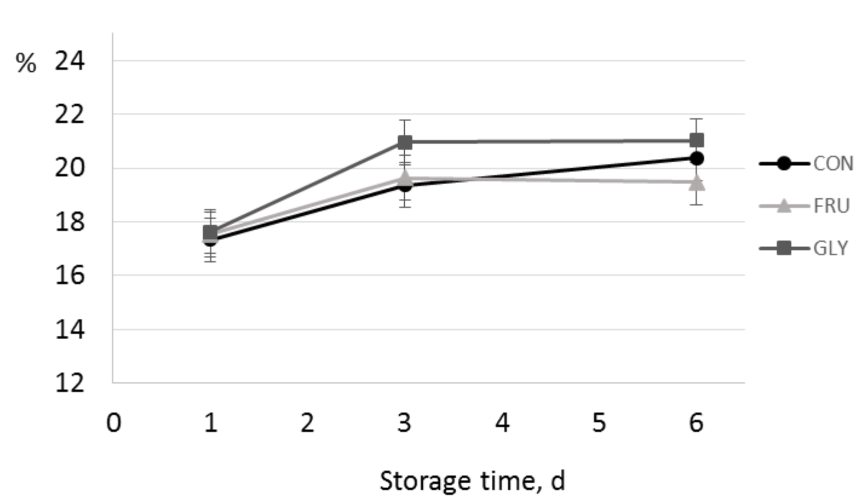


Figure 3.7. Expressible moisture of *Longissimus* muscle storage at 4°C for 1, 3, or 6 d of lambs offered regular drinking water (CON) or supplemented with glycerol (GLY) or fructose (FRU) via drinking water. Expressible moisture was not affected by treatment (P = 0.51) but was affected by aging time (P = 0.0003). The interaction between treatment and aging time was nonsignificant (P = 0.80).

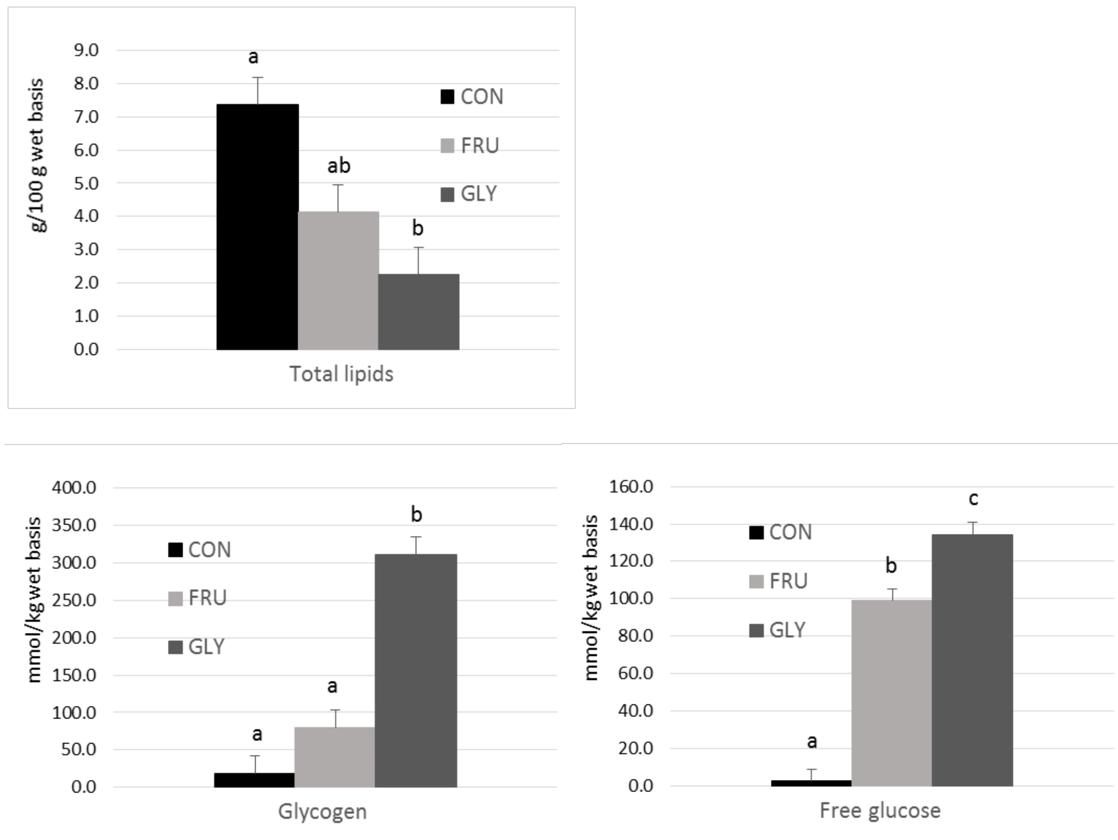


Figure 3.8. Total lipids, glycogen, and free glucose concentration of liver at 30 min postmortem of lambs offered regular drinking water (CON) or supplemented with glycerol (GLY) or fructose (FRU) via drinking water. Liver glycogen, free glucose and total lipids were affected by treatment ($P < 0.05$). [a,b]Means with different letters are significantly different ($P < 0.05$).

Table 3.6. *Longissimus* fatty acid profile of lambs offered regular drinking water (CON) or supplemented with glycerol (GLY) or fructose (FRU) via drinking water.

	CON	FRU	GLY	SEM	P-level
TFA[1], g/100g wet basis	3.23	3.28	2.54	1.027	0.355
Fatty acid profile, %					
C14:0	2.21	2.30	2.35	0.179	0.875
C15:0	0.21	0.35	0.38	0.078	0.349
C16:0	22.78	23.73	22.90	0.920	0.718
C16:1 *cis*-9	1.30	1.61	1.54	0.078	0.108
C17:0	1.19[a]	1.16[a]	2.03[b]	0.127	**0.036**
C18:0	20.85[y]	17.27[x]	16.89[x]	0.810	**0.064**
C18:1 *trans*-10	0.29	0.49	0.16	0.194	0.653
C18:1 *trans*-11	1.68	1.59	1.80	0.183	0.758
C18:1 *cis*-9	39.15	40.74	41.31	0.757	0.254
C18:1 *cis*-11	0.59	1.01	1.26	0.313	0.430
C18:2 *cis*-9,12	4.05	3.63	3.63	0.273	0.499
C18:2 *cis*-9, *trans*-11	0.47	0.53	0.45	0.041	0.427
C18:2 *trans*-10, *cis*-12	0.04	0.05	0.01	0.018	0.403
C18:3 *cis*-9,12,15	0.78	0.78	0.77	0.065	0.996
C20:4 *cis*-5,8,11,14	1.66	1.74	1.95	0.183	0.631
C20:5, EPA	0.19	0.27	0.25	0.029	0.224
C22:5, DPA	0.37	0.40	0.44	0.033	0.491
C22:6, DHA	0.08[x]	0.12[xy]	0.19[y]	0.018	**0.057**
SFA	45.84	43.31	42.14	0.978	0.153
OCFA	1.40[a]	1.51[a]	2.41[b]	0.160	**0.047**
MUFA	40.45	42.35	42.85	0.743	0.189
PUFA n-6	5.71	5.36	5.57	0.420	0.826
PUFA n-3	1.42	1.57	1.66	0.116	0.464
Ratio n-6:n-3	4.09	3.47	3.38	0.361	0.400

[1]TFA: Total fatty acids.
[a,b]Means in a row followed by different superscripts differ ($P < 0.05$).
[x,y]Means in a row followed by different superscript differ ($P < 0.10$).

Table 3.7. Subcutaneous fatty acid profile of lambs offered regular drinking water (CON) or supplemented with glycerol (GLY) or fructose (FRU) via drinking water.

	CON	FRU	GLY	SEM	P-level
TFA[1], g/100g wet basis	74.78	81.29	71.28	5.297	0.474
Fatty acid profile, %					
C14:0	2.73	2.94	3.20	0.309	0.656
C15:0	0.65[x]	0.61[x]	0.88[y]	0.047	**0.059**
C16:0	22.10	24.30	22.29	1.093	0.366
C16:1 *cis*-9	1.11	1.38	1.27	0.085	0.183
C17:0	2.62	2.35	4.01	0.535	0.245
C18:0	28.74[b]	23.07[a]	24.33[a]	0.702	**0.018**
C18:1 *trans*-10	0.24	0.65	0.25	0.218	0.385
C18:1 *trans*-11	3.92	3.45	2.92	0.411	0.403
C18:1 *cis*-9	30.68[x]	34.09[y]	34.28[y]	0.733	**0.066**
C18:1 *cis*-11	0.67	0.53	0.26	0.268	0.643
C18:2 *cis*-9,12	2.16	1.72	1.36	0.406	0.301
C18:2 *cis*-9, *trans*-11	0.64	0.75	0.75	0.064	0.456
C18:2 *trans*-10, *cis*-12	0.05	0.04	0.05	0.012	0.884
C18:3 *cis*-9,12,15	0.75	0.70	0.61	0.059	0.396
C20:4 *cis*-5,8,11,14	0.11	0.10	0.11	0.011	0.941
C22:5, DPA	0.09	0.09	0.09	0.005	0.992
C22:6, DHA	0.02	0.01	0.02	0.005	0.730
SFA	53.32	50.30	49.88	1.862	0.443
OCFA	3.27	2.96	4.89	0.583	0.217
MUFA	31.79[x]	35.47[y]	35.55[y]	0.741	**0.057**
PUFA n-6	2.27	1.83	1.46	0.298	0.319
PUFA n-3	0.87	0.81	0.72	0.058	0.382
Ratio n-6:n-3	2.96	2.46	2.13	0.491	0.575

[1]TFA: Total fatty acids.
[a,b]Means in a row followed by different superscript differ ($P < 0.05$).
[x,y]Means in a row followed by different superscript differ ($P < 0.10$).

Table 3.8. Liver fatty acid profile of lambs offered regular drinking water (CON) or supplemented with glycerol (GLY) or fructose (FRU) via drinking water.

	CON	FRU	GLY	SEM	P-level
TFA[1], g/100g wet basis	5.92[b]	3.14[a]	1.63[a]	0.592	**0.032**
Fatty acid profile, %					
C14:0	0.85[x]	0.82[x]	1.11[y]	0.056	**0.080**
C15:0	0.40[a]	0.40[a]	0.78[b]	0.007	**<0.0001**
C16:0	19.30[b]	16.67[a]	17.63[ab]	0.430	**0.039**
C16:1 *cis*-9	1.20	1.80	2.20	0.232	0.123
C17:0	1.25	1.61	2.88	0.194	0.856
C18:0	18.57[x]	19.65[xy]	22.28[y]	0.700	**0.085**
C18:1 *trans*-10	0.15	0.30	0.10	0.102	**0.472**
C18:1 *trans*-11	2.07[y]	2.26[y]	0.95[x]	0.266	**0.092**
C18:1 *cis*-9	26.81	22.90	20.23	1.653	0.149
C18:1 *cis*-11	0.79[a]	1.69[b]	1.59[b]	0.161	**0.046**
C18:2 *cis*-9,12	8.23[b]	5.31[a]	3.72[a]	0.508	**0.007**
C18:2 *cis*-9, *trans*-11	1.33	1.13	0.82	0.105	0.104
C18:2 *trans*-10, *cis*-12	0.13[a]	0.22[a]	0.44[b]	0.048	**0.049**
C18:3 *cis*-9,12,15	2.38[b]	1.49[a]	1.01[a]	0.187	**0.031**
C20:4 *cis*-5,8,11,14	5.19	7.01	6.67	0.452	0.101
C20:5, EPA	1.48[a]	2.42[b]	2.66[b]	0.195	**0.044**
C22:5, DPA	2.81[a]	4.98[b]	4.77[b]	0.360	**0.034**
C22:6, DHA	1.87[x]	2.56[xy]	3.46[y]	0.246	**0.050**
SFA	38.72[a]	37.14[a]	41.01[b]	0.464	**0.026**
OCFA	1.65[a]	2.01[a]	3.67[b]	0.192	**0.013**
MUFA	28.01	24.70	22.43	1.683	0.216
PUFA	21.97	23.77	22.28	0.921	0.395
PUFA n-6	13.43	12.32	10.39	0.701	0.136
PUFA n-3	8.54[a]	11.45[b]	11.89[b]	0.600	**0.047**
Ratio n-6:n-3	1.60[b]	1.10[a]	0.85[a]	0.091	**0.022**

[1]TFA: Total fatty acids.
[a,b]Means in a row followed by different superscript differ ($P < 0.05$).
[x,y]Means in a row followed by different superscript differ ($P < 0.10$).

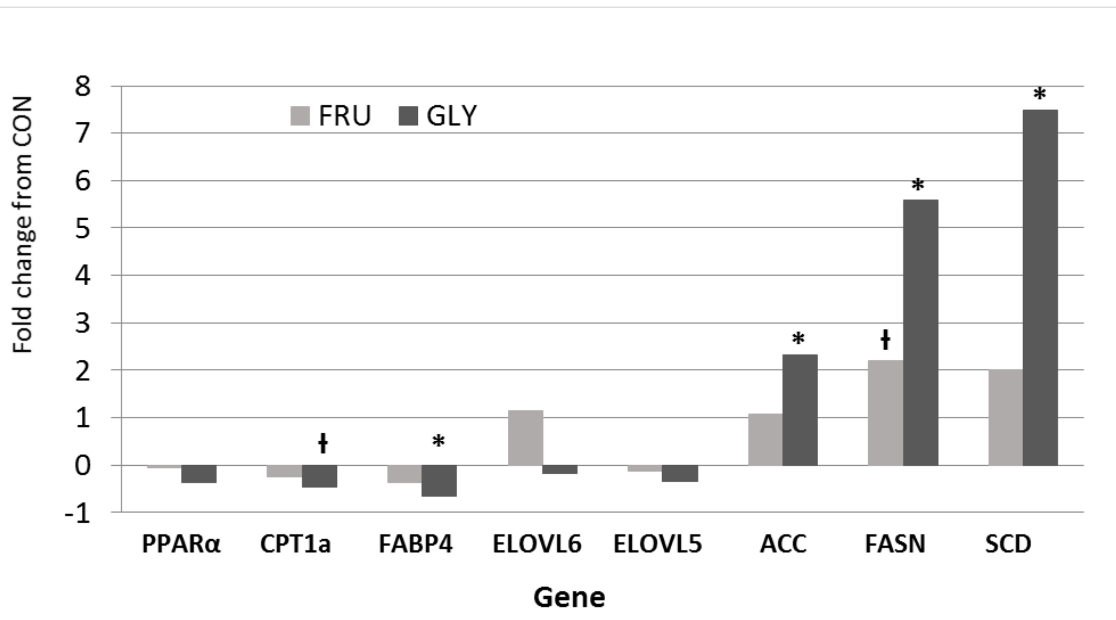


Figure 3.9. Relative expression of genes associated with lipid metabolism in liver of lambs offered regular drinking water (CON) or supplemented with glycerol (GLY) or fructose (FRU) via drinking water. PPARα: peroxisome proliferator-activated receptor-α, CPT1a: carnitine palmitoyltransferase 1a, FABP4: fatty acid binding protein 4, ELOVL6: fatty acid elongase-6, ELOVL5: fatty acid elongase-5, ACC: acetyl-CoA carboxylase, FASN: fatty acid synthase, SCD1: stearoyl-CoA desaturase-1. *Gene expression differ (P < 0.05) from CON. † Gene expression tended to differ (P < 0.10) from CON.

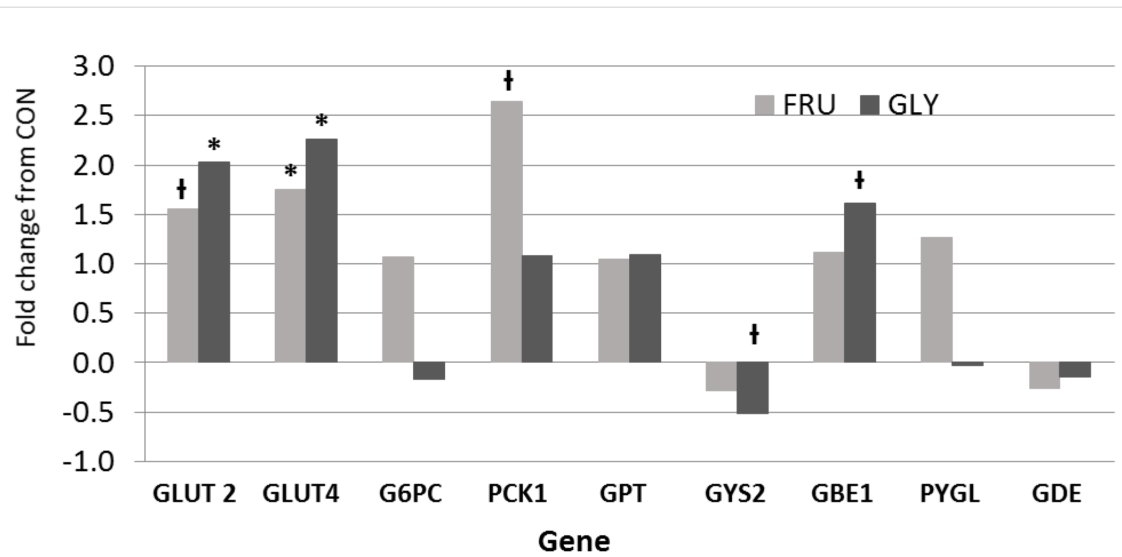


Figure 3.10. Relative expression of genes associated with glucose metabolism in liver of lambs offered regular drinking water (CON) or supplemented with glycerol (GLY) or fructose (FRU) via drinking water. GLUT2: facilitated glucose transporter member 2, GLUT4: facilitated glucose transporter member 4, G6PC: glucose-6-phosphatase, PCK1: phosphoenol pyruvate carboxykinase, GPT: glutamic-pyruvate transaminase, GYS2: glycogen synthase 2, GBE1: glycogen branching enzyme, PYGL: glycogen phosphorylase, GDE: glycogen debranching enzyme. *Gene expression differ ($P < 0.05$) from CON. † Gene expression tended to differ ($P < 0.10$) from CON.

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