# Glucose Metabolism in Low Birth Weight Neonatal Pigs

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## Abstract

The neonatal period in mammals is characterized by high growth rates and is dominated by skeletal muscle hypertrophy. Low birth weight (LBWT) neonates experience restricted growth and development of skeletal muscle, leading to metabolic perturbations later in life. The overall hypothesis of this dissertation was that in utero disturbances in glucose metabolism and increased energy requirements predisposes LBWT neonatal pigs to metabolic disturbances after birth. We sought to increase growth of skeletal muscle and improve glucose production through increasing dietary energy and to determine the changes in glucose catabolism and metabolic flexibility in different skeletal muscle fiber types in LBWT neonates. Piglets were considered normal birth weight (NBWT) and LBWT when birth weight was within 0.5 SD and below 2 SD of the litter average, respectively. Increasing dietary energy increased lean deposition in the *longissimus dorsi* (LD) in both NBWT and LBWT neonates. Although glucose rate of appearance was greater in LBWT compared to their NBWT sibling, glucose concentrations were reduced in LBWT compared to NBWT pigs, regardless of diet fed. Postprandial glucose concentrations were lower in LBWT compared to NBWT pigs, regardless of diet fed, although rate of appearance did not differ between them. This would suggest that glucose is being absorbed in the peripheral tissues to be utilized. However, expression of enzymes related to glycolysis were downregulated in both the soleus and LD of LBWT compared to NBWT neonatal pigs. In addition, expression of enzymes related to the catabolism of glucose in the serine biosynthetic pathway were decreased in both the soleus

and LD muscles of LBWT compared to NBWT neonatal pigs. Expression of the pentose phosphate pathway was slightly increased in LBWT compared to NBWT siblings in both muscle types. Increased expression of *pyruvate dehydrogenase 4* was exhibited in both the soleus and LD of LBWT pigs compared to NBWT siblings. This would indicate a switch in fuel utilization to more fatty acid oxidation. By contrast, $CO_2$ production from the oxidation of palmitate was reduced in LBWT compared with NBWT pigs along with reduced oxidation of glucose and pyruvate. In conclusion, lipid supplementation increased growth at the expense of fat deposition in the liver of NBWT and LBWT pigs. However, supplementing with fat did not increase glucose production due to the contribution of glycerol remaining constant. Hypoglycemia cannot be attributed to greater catabolism in skeletal muscle due to decreased expression of glycolytic genes and the addition of fatty acids did not spare glucose oxidation in skeletal muscle of LBWT pigs.

**Keywords:** Low birth weight, pig, neonate, skeletal muscle, glucose metabolism, fatty acid metabolism

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## General Audience Abstract

During the neonatal period animals display the fastest growth rates, especially pertaining to muscle growth. Muscle development in low birth weight (LBWT) is restricted, leading not only to impaired postnatal growth but increases the risk for developing metabolic diseases later in life such as obesity and type 2 diabetes. LBWT is also characterized by decreased glucose concentrations and decreased body fat content at birth. In the present studies we sought to increase growth and improve glucose production by supplementing with a high energy diet and to compare the changes in glucose catabolism in different skeletal muscle fiber types along with analyzing the ability to switch fuel substrates in LBWT and NBWT neonatal pigs. Increasing dietary energy increased *longissimus dorsi* (LD) weight as a percentage of bodyweight, regardless of growth status. In addition, during fasting glucose production was higher in LBWT compared to their NBWT siblings, regardless of diet. However, glucose concentration in LBWT were lower compared to NBWT neonatal pigs. Although glucose concentrations were lower in LBWT compared to NBWT pigs after a meal, glucose production rate was unchanged among LBWT and NBWT siblings fed either a high or low energy diet. This suggests that glucose uptake is increased in peripheral tissues of LBWT pigs. However, enzymes related to glycolysis in the LD and soleus of LBWT pigs had lower expression than their NBWT sibling. In addition, the enzyme responsible for the shift in fuel selection, pyruvate dehydrogenase kinase 4 (PDK4) was highly expressed in LBWT compared to NBWT neonatal pigs in both the LD and soleus. This would suggest a switch in glucose oxidation

to fatty acid oxidation in the skeletal muscle of LBWT neonatal pigs. However, oxidation of fatty acids in both the soleus and LD of LBWT was reduced compared to NBWT neonatal pigs. In conclusion, lipid supplementation increased growth at the expense of lipid deposition in the liver and did not increase glucose production. Reduced glucose concentrations are not due to greater catabolism in skeletal muscle due to decreased expression of glycolytic genes and the addition of fatty acids did not spare glucose oxidation in the skeletal muscle of LBWT pigs.

**Keywords:** low birth weight, glucose metabolism, lipid intake, fatty acid metabolism, skeletal muscle

## Dedication

This dissertation is dedicated to my parents Glenn and Kerry McCauley. Their hard work, sacrifice and constant support has allowed me to dream big and continuously make them a reality.

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## Introduction

Low birth weight remains a problem in perinatal medicine and animal production. Infants born weighing less than the 10$^{th}$ percentile (Rosenberg, 2008a) are clinically considered low birthweight (LBWT). LBWT is not only a problem in developing countries where up to ~25% of infants are affected, but also in developed countries like the U.S. where 10 to 15% of infants are affected (Berghella, 2007; Saleem et al., 2011). LBWT infants are at a higher risk of perinatal mortality as well as short- and long-term childhood morbidities, and metabolic disorders, including insulin resistance, type 2 diabetes, cardiovascular disease, and obesity later in life (Barker, 2000; Godfrey and Barker, 2000; Resnik, 2002). LBWT not only decreases the chances of survival but also has permanent stunting effects (Widdowson, 1971; Bauer et al., 2003; Wu et al., 2006).

Infancy is characterized as the period where the body displays the highest growth rate compared to any other period in the postnatal life (Reeds et al., 2000). During this period, growth is dominated by skeletal muscle hypertrophy (Schiaffino et al., 2013). The LBWT human neonates is characterized by asymmetrical growth, resulting in lower length of body in relation to normal head circumference (Lubchenco et al., 1966). Asymmetrical growth accounts for up to 75% of the LBWT human infants (Ferenc et al., 2014). In pigs, the prevalence of natural LBWT is high due to selection for large litter size. LBWT accounts for up to 15% of the litter, greater than any other livestock animal (Quiniou et al., 2002). One characteristic of growth in spontaneously occurring LBWT pigs is the disproportionate growth of skeletal muscle compared to other organs (Bauer et al., 2003; Ferenc et al., 2014). In addition, due to skeletal muscle accounting for a large proportion of bodyweight and significant part in whole body homeostasis (Yates et al., 2012),

decreases in skeletal muscle development and function may ultimately lead to not only impaired growth but long-term metabolic disturbances later in life (Brown, 2014). Due to large physiological similarities between species, the LBWT piglet is considered as a suitable model for low birth weight infants (Bauer et al., 2003). Gaps in our knowledge exist in relation to the metabolic changes that occur in LBWT neonates. Understanding such changes may be beneficial to maximizing growth and improving health outcomes.

Two observations are of special interest to this research. The first is that blood glucose concentration is lower in LBWT neonatal pigs compared to their NBWT siblings (Chen et al., 2017). Glucose is a primary fuel source for metabolism in most muscles, organs, and tissues in the body; this is why hepatic glucose production is critical for successful transition from intrauterine to extra-uterine life. After birth, a decrease in plasma insulin concentration levels and an initial surge in glucagon concentration occurs (Sperling et al., 1974). Due to limited stores of glycogen in the liver, there is a need for glucose production from other sources. This creates endocrine and metabolic responses, driving an increase in hepatic glycogenolysis, lipolysis, and proteolysis (Platt and Deshpande, 2005). Thus, the contribution of amino acids, lactate and glycerol to gluconeogenesis (GNG) becomes more significant (Platt and Deshpande, 2005). These metabolic changes occur to counter normal hypoglycemia that occurs after the umbilical cord is severed. However, LBWT neonates are at a greater risk for disturbed glucose homeostasis due to limited substrate availability, such as depletion of hepatic glycogen reserves, and the increase in metabolic demands due to a relatively larger brain size (Platt and Deshpande, 2005; Chacko et al., 2011). In addition, LBWT neonates have a decreased phosphoenolpyruvate carboxykinase activity, limited mobilization and subsequent

oxidation of fatty acids, functional hyperinsulinemia, and potentially immature regulation of glucose metabolism (Platt and Deshpande, 2005; Chacko et al., 2011).

The second observation is that the reduction of muscle mass, coupled to a shift in muscle oxidative capacity in LBWT neonates could influence glucose metabolism. Skeletal muscle accounts for up to 40% of muscle mass in the body and serves as a regulator for energy and protein metabolism making it a key site for glucose uptake and utilization (Meyer et al., 2002). Due to the fact that muscle accounts for the majority insulin-stimulated glucose uptake and catabolism (DeFronzo et al., 1981), it is also considered to be the initiating or primary defect in insulin resistance (Lillioja et al., 1988; Warram et al., 1990). The current concept to explain these outcomes is that insulin resistance in tissues is a result of increased concentrations of plasma insulin in an attempt to clear high amounts of glucose associated with obesity (Wilcox, 2005). In obese individuals, skeletal muscle exhibits a shift in fiber-type by having a reduced oxidative capacity to increased glycolysis (Tanner et al., 2002; Oberbach et al., 2006). In addition, there is a negative relationship between the relative percentage of type 1 fibers and obesity (Lillioja et al., 1987; Hickey et al., 1995). Similar changes are presented in individuals with type 2 diabetes (Marin et al., 1994; Tanner et al., 2002). Changes in fiber type are also associated with LBWT (Wigmore and Stickland, 1983; Jensen et al., 2007). Due to stunted growth LBWT individuals have lower muscle mass and have been shown to have reduced oxidative capacity and increased glycolytic capacity (Jensen et al., 2007; Jensen et al., 2008). Impaired growth of skeletal muscle could be a major contributor to lifelong insulin resistance and glucose intolerance (Brown, 2014).

Having a better understanding of the role that glucose metabolism plays in NBWT and LBWT neonates can help design new strategies to alleviate the effects of IUGR in neonates. The overall hypothesis of this dissertation is that low glucose production and high catabolism of glucose in skeletal muscle predisposes LBWT neonatal pigs to slower postnatal growth. The objectives of this project were to: 1) Investigate the changes in growth performance and body composition when dietary energy was increased, 2) Determine changes in glucose and glycerol kinetics when dietary energy was increased, 3) Profile changes in glucose catabolic pathways of skeletal muscle, 3) Examine the changes in glucose catabolic pathways in oxidative and glycolytic muscle types and 4) Determine changes in substrate oxidation in skeletal muscle.

# Chapter 1 Literature Review

## Low Birth Weight Neonates

### *Causes of LBWT*

Intrauterine growth restriction (IUGR) is defined as impaired growth and development of the mammalian fetus or its organs during pregnancy. IUGR is one of the major factors contributing to LBWT (Rosenberg, 2008a). Genetic, epigenetic and environmental factors play a large role in fetal growth and development and ultimately birth weight (Wu et al., 2006).

Evidence states that during pregnancy adverse events can be inherited across generations. The effects of early life programming are not only seen in offspring of which the event occurred but also in the next generation. For example, positive correlations have been found between birthweights of infants and the birth weight of their parents (Emanuel et al., 1992; Harrison and Langley-Evans, 2009). Intergenerational effects on glucose metabolism are seen in the offspring of LBWT (Martin et al., 2000; Srinivasan et al., 2003). Maternal low protein diet in rats reduces insulin secretion in the F1 generation while the F2 generation exhibited insulin resistance (Benyshek et al., 2006). Others have demonstrated that F1 and F2 generations of maternal undernutrition experienced LBWT, impaired glucose tolerance and obesity with age (Jimenez-Chillaron et al., 2009).

In addition to genetic defects, epigenetic changes in gene expression have led to increased susceptibility to metabolic diseases in LBWT (Hales and Barker, 1992; Waterland and Jirtle, 2004). Epigenetic modification refers to DNA modifications that result in differential gene expression without altering genomic sequence. These modifications include DNA methylation, genomic imprinting and chromatin modifications

such as post translational modifications of histones. Epigenetic modifications affecting glucose metabolism have been described in IUGR liver and skeletal muscle. For example, maternal protein restriction decreased methylation of genes associated with glucocorticoid receptor in the liver of offspring (Jing-Bo et al., 2013). In addition, decreased methylation of genes for peroxisome proliferator-activated receptor alpha (PPARα) occurs in the liver of IUGR rats at weaning (Jing-Bo et al., 2013). In skeletal muscle of LBWT, isoforms of transcription factor MEF2 that binds to GLUT4 promoter form heterodimers and ultimately reduce *glut4* gene expression (Raychaudhuri et al., 2008). In addition, epigenetic changes to the transcription factor pancreatic and duodenal homeobox 1 which is responsible for β-cell development and function, is decreased in LBWT neonatal rats and leads to increased susceptibility of diabetes later in life (Pinney et al., 2011). Imprinting genes, such as *IGF-II*, enhance growth of the fetus and exhibited hypomethylation in individuals who were malnourished in utero (Heijmans et al., 2008). Histone modification and DNA methylation are distinctively regulated in the liver of LBWT rats consistent with the changes in hepatic gene expression (MacLennan et al., 2004). Altogether, adaptations that are induced prenatally have important implications for metabolic programming.

The environment in utero is another factor that plays an important role in fetal growth and development. In polytocous species the position of the fetus in the uterus may affect development. For example, in pigs fetuses located at either end of the uterine horns are generally larger than those located more centrally (Perry and Rowell, 1969). Furthermore, litter bearing species show reduced placental mass per fetus and ultimately placental insufficiency due to the large number of fetuses (Wootton et al., 1983; Redmer et al., 2004). In species such as sheep, cattle and horses, embryos transferred to a dam of

lower uterine capacity results in LBWT offspring (Dickinson et al., 1962; Ferrell, 1991; Allen et al., 2002).

Maternal nutrition also affects the uterine environment, such as under- and over-nutrition (Wu et al., 2006). In livestock species restricted nutrient supply due to limited forage and feedstuff during gestation negatively impacts fetal growth and development (Gonzalez et al., 2013). Pigs exhibit a natural under-nutrition due to disproportionate supply of maternal nutrients in the uterine horns (Dwyer et al., 1994). A low protein diet before and during pregnancy in rats have adverse effects on glucose metabolism in offspring (Harrison and Langley-Evans, 2009). In humans, limited nutrient supplies during gestation lead to maternal undernutrition and cause adverse growth effects of the fetus (Veenendaal et al., 2011). In addition, impaired transfer of nutrients to the fetus caused by placental insufficiency contributes to LBWT in neonates (Fowden et al., 2006; Jansson and Powell, 2007).

Along with maternal undernutrition, over nutrition during pregnancy increases the risk for reduced fetal growth and neonatal morbidity and mortality (Castro and Avina, 2002; Wallace et al., 2003). Diet induced obesity in gestating sheep causes an increase in triglyceride levels in the fetus, thus increasing placental inflammation and negatively affecting fetal growth (Zhu et al., 2010). In addition, over-nourished sheep cause defects in skeletal muscle development and increased fat deposition in offspring (Tong et al., 2009; Yan et al., 2010). In pigs, overfeeding by 40% during gestation results in impaired growth and development of the fetus and reduced postnatal survival (Han et al., 2000). Maternal obesity in humans is becoming an epidemic and is harmful for fetal development (Tenenbaum-Gavish and Hod, 2013; Marchi et al., 2015). In addition, maternal obesity

predisposes the offspring to metabolic disturbances later in life, such as obesity and diabetes (Du et al., 2010).

*Fetal programming*

Adaptation of the fetus to adverse stimuli during development is known as fetal programming (Lucas, 1991, 1994; Barker, 1998). Fetuses that endure IUGR, in both humans and in livestock, alter metabolic homeostasis which increases the risk for developing metabolic diseases later in life (Yates et al., 2012). "Thrifty phenotype" was the first theory to explain the development of metabolic diseases as a consequence of fetal programming. This theory suggests that poor early life nutrition produces adaptive changes in the fetus in order to maintain growth of key essential organs (such as the brain) at the expense of other organs (such as the pancreas). In turn, these adaptations produce permanent changes in glucose-insulin metabolism (Hales and Barker, 2001).

There is an association between early nutritional environment, postnatal growth and the development of metabolic diseases and altered fat deposition in adult life (Fall et al., 1995; Loos et al., 2002; Cripps et al., 2005). In mice and rat models of maternally induced LBWT, skeletal muscle alterations suggest that adverse effects *in utero* induces permanent metabolic changes (Selak et al., 2003; Raychaudhuri et al., 2008; Huber et al., 2009). These metabolic diseases linked with impaired fetal growth and development may be due to endocrine dysfunction during fetal development which could result in atypical hormone bioavailability later in life (Fowden et al., 2005). For example, during times of nutrient restriction during late gestation in ewes anabolic hormones such as insulin, insulin-like growth factor 1, thyroxine concentrations are reduced and catabolic hormones such as cortisol, catecholamines and growth hormone are greater (Fowden and Forhead, 2004). In

addition, inadequate supply of nutrients to the fetus during late gestation causes chronic fetal hypoglycemia and hypoxemia resulting in adverse effects on development and function of endocrine glands such as pancreatic islets (Hoet and Hanson, 1999; Fowden et al., 2005; Yates et al., 2012). These changes in late gestation lead to increased risk for the development of metabolic diseases with aging (Yates et al., 2012).

*Animal models of LBWT*

Research using animal models for LBWT have provided extensive insight and basis for the findings for human studies. One of the first models of placental insufficiency was produced by uterine artery ligation (Wigglesworth, 1964). The most common animal models of LBWT are induced by restricting maternal calories or protein diets in mice and rats. Although rodents and humans share similar genes and physiology, the brain and endocrine system are not mature until the weaning period, likely making it a limitation for their use (Vuguin, 2007). Along with rodent models, using sheep for placental insufficiency studies have been used. In pregnant sheep, LBWT has been induced by several factors such as maternal over- and under-nutrition, utero-placental embolization, uterine carunclectomy, single umbilical artery ligation and administration of glucocorticoids (Oyama et al., 1992; Wallace et al., 1999; Moss et al., 2001; Morrison, 2008). Although there has been extensive work using sheep as a model for LBWT, many of the results are variable across breed and intervention used (Anthony et al., 2003). Primates are the ideal model to use however their use is largely restricted due to ethical considerations. Pigs also serve as a model for human infants due to metabolic and physiological similarities. In addition, the development of LBWT in pigs, like humans, is spontaneous and does not require maternal diet manipulation or other experimental

interventions (Ferenc et al., 2014). Spontaneously occurring LBWT in pigs is asymmetrical in that there is disproportionate growth of skeletal muscle and internal organs in relation to the brain (Bauer et al., 2003). These similarities in spontaneously occurring LBWT pigs are characteristic and indicative of the adverse effects that accompany asymmetrical LBWT in humans (Lubchenco et al., 1966), suggesting the naturally occurring LBWT in pigs is a suitable model for LBWT infants.

*LBWT effects on skeletal muscle*

The majority of muscle fibers are formed during the prenatal stage in animals. Prenatal myogenesis can be divided into primary and secondary myogenesis. Primary myogenesis is when primary fibers arise and occurs mainly during the embryonic stage which serves as a scaffolding for secondary myogenesis (Picard et al., 2002). Secondary myogenesis leads to the formation of secondary fibers which comprise the majority of myofibers and are higher nutrient responsive (Ward and Stickland, 1991; Dwyer et al., 1994). Secondary myogenesis occurs mainly during the fetal stage (Russell and Oteruelo, 1981). Several studies of maternal nutrient restriction during pregnancy in pigs and sheep show the dramatic effects on fetal myofiber number in that the ratio of secondary to primary fibers is lower (Handel and Stickland, 1987; Ward and Stickland, 1991; Dwyer et al., 1994; Zhu et al., 2004). The reduced ratio of secondary to primary fibers could possibly lead to decreased nutrient responsiveness. In addition, LBWT pigs have decreased number of skeletal muscle fibers that cannot be altered after birth (Rehfeldt and Kuhn, 2006)

Altered development of skeletal muscle fibers in LBWT has consequences on glucose uptake, insulin signaling and oxidative capacity in neonates and adults. Muscle fibers are categorized into two broad categories of type I and type II. Type II fibers are

further categorized as type IIa, IIx and IIb. Type I fibers are more oxidative or aerobic, higher in mitochondria numbers and have greater myoglobin content (Pette and Staron, 2000). In contrast, type II fibers are more glycolytic with lower mitochondria numbers and myoglobin content (Pette and Staron, 2000). Within type II fibers however, there is variation in metabolism with type IIa expressing more oxidative potential than type IIb which are more glycolytic and IIx are intermediate of the two (Zierath and Hawley, 2004). Low birth weight fetal sheep have a shift from type 1 fiber type to type II (Yates et al., 2016). In pigs however at birth, LBWT piglets exhibit more oxidative compared to glycolytic fibers (Pardo et al., 2013) although at market these pigs have a switch to a more glycolytic fiber phenotype (Bee, 2004). This shift in adulthood is also seen in humans which is associated with insulin resistance (Jensen et al., 2007). When challenged with a hyperinsulinemia-euglycemic clamp, LBWT adults experience decreased glucose uptake (Jaquet et al., 2000). In addition, local infusion of insulin resulted in reduced muscle glucose uptake in LBWT adults along with changes in insulin signaling proteins and GLUT4 expression compared to NBWT healthy adults (Hermann et al., 2003; Ozanne et al., 2005; Jensen et al., 2007).

*LBWT transition from intrauterine to extrauterine life*

After birth but before suckling is established in NBWT humans and animals, a surge in glucagon to mobilize glycogen is present to counteract decreased glucose and insulin levels (Sperling et al., 1974). Due to limited glycogen stores in the liver, glucose production from non-carbohydrate sources are increased generating substrates that are utilized in gluconeogenesis (GNG) (Platt and Deshpande, 2005). LBWT human neonates exhibit hypoglycemia after birth owing in part to decreased glycogen stores compared to

NBWT and increased metabolic demands due to larger brain size in relation to other organs (Rosenberg, 2008b; Chacko et al., 2011). Total parenteral nutrition (TPN) is prescribed to LBWT infants in an attempt to increase glucose production and help facilitate growth and development. However, in LBWT neonates administered TPN they have an increased risk for hyperglycemia (Cowett et al., 1979). In addition, the rate limiting enzyme for GNG, phosphoenolpyruvate carboxykinase (PEPCK) activity is diminished in LBWT neonatal rats causing a delayed onset of GNG (Pollak et al., 1979). At weaning LBWT pigs have increased expression of PEPCK compared to NBWT pigs (Liu et al., 2012). Gluconeogenic substrates such as alanine and lactate are increased in LBWT neonates (Haymond et al., 1974), however they do not differ in response of oral administration of alanine in LBWT compared to NBWT neonates (Williams et al., 1975; Frazer et al., 1981) consistent with decreased PEPCK activity. However, glycerol turnover through lipolysis can account for up to 80% of glucose production in the neonate (Patel and Kalhan, 1992; Chacko and Sunehag, 2010). Increasing dietary fat in newborn infants (Sann et al., 1988; Bougneres et al., 1989) and pigs (Le Dividich et al., 1991) have exhibited increased plasma glucose concentrations. Altogether, glucose production is an important metabolic fuel source and having a better understanding of the role that it plays in LBWT neonates can help alleviate the metabolic dysregulation that occurs later in life.

**Energy Metabolism**

Dietary energy consumption is needed to support the energy expenditure demands by the body. This energy is utilized by numerous biological processes such as growth, tissue repair, muscle contraction and maintenance. Energy consumed is mainly supplied by macronutrients like carbohydrates, lipids and proteins. Once digested, macronutrients

are broken down to simpler molecules and transported throughout the body to be utilized. When dietary carbohydrate, lipids and proteins are provided in excess of the body's immediate need they are stored as glycogen, triglycerides, or converted to glucose, respectively.

## Glucose Metabolism

### *Glucose absorption and utilization*

Typical Western diets consists of one that is high in saturated fats, red meats, 'empty' carbohydrates and low in fruits in vegetables, whole grains, seafood and poultry(McGraw-Hill, 2002). Complex dietary carbohydrates must be hydrolyzed to monosaccharides, such as glucose or galactose, in the small intestine. Simpler forms of the complex carbohydrates make it easier to cross intestinal brush-border membrane, primarily mediated by sodium glucose transporter SGLT1. Transport out of the enterocytes is facilitated by GLUT2 passive transport. This transporter allows glucose to move from the small intestine epithelial cells into blood circulation (Roder et al., 2014). Glucose is transported by the bloodstream to tissues such as the liver, brain and skeletal muscle to be catabolized for energy or for storage.

Utilization of glucose by the liver represents one third of an oral glucose load (Ferrannini et al., 1985; Mari et al., 1994). Absorption of glucose by the liver occurs through the non-insulin dependent glucose transporter GLUT2 which also mediates the release of glucose along with GLUT1 (Seyer et al., 2013). In the postprandial state a majority of the glucose consumed by the liver is stored as glycogen or catabolized to produce ATP (Agius, 2008). During fasting glucose released from glycogen through glycogenolysis to generate glucose (Kotoulas et al., 2004). Along with the liver the brain

also absorbs glucose in a non-insulin like manner across the blood brain barrier through glucose transporter GLUT1 and GLUT3 (Simpson et al., 2007). The brain represents ~2% of bodyweight yet consumes ~20% of glucose derived energy (Erbsloh et al., 1958), making it the major consumer of glucose.

Most insulin-stimulated glucose uptake takes place in the skeletal muscle and adipose tissue and accounts for ~80% of post prandial glucose uptake and utilization (DeFronzo et al., 1985; Ferrannini et al., 1988). Glucose is mainly transported across the cell membrane by glucose transporter 4 (GLUT4). In response to an increase in insulin, GLUT4 is translocated to the cell membrane where glucose is then transported into the cells (Charron et al., 1989; Kahn, 1992; Huang and Czech, 2007). Glucose is mainly catabolized to ATP in skeletal muscle for contraction but can also be stored as glycogen to be used for local energy support during "fight or flight" situations (Jensen et al., 2011).

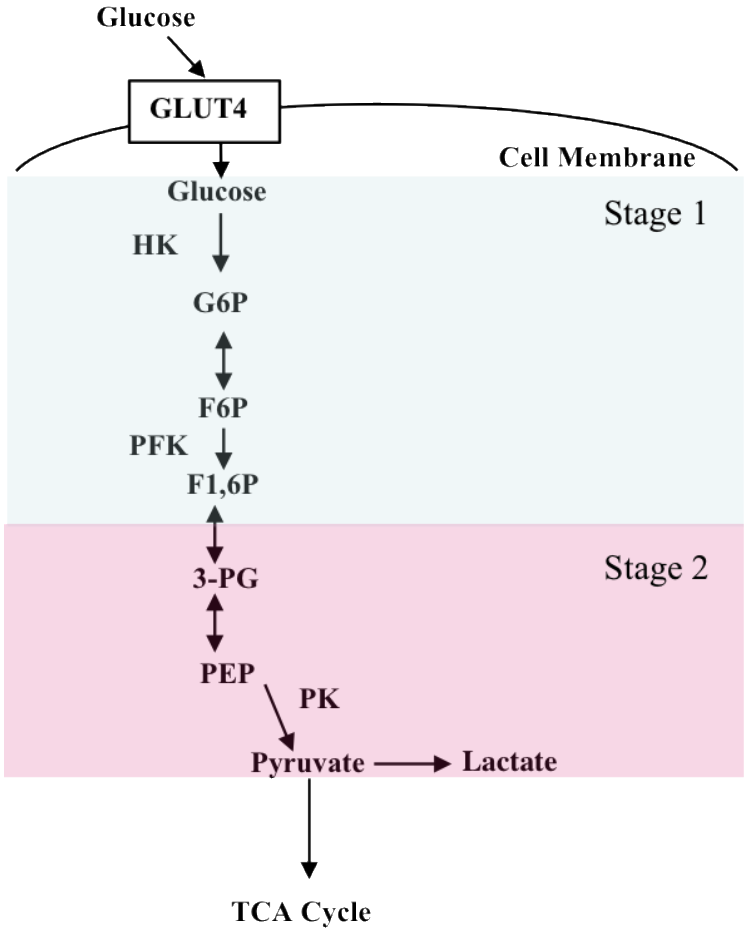


**Figure 1-1.** Glycolysis pathway. Anaerobic glycolysis is split into two stages. Stage 1) Phosphorylation of glucose and its conversion to glyceraldehyde 3-phosphate. Stage 2) Conversion of glyceraldehyde 3-phosphate to pyruvate and the formation of ATP.

*Glycolysis*

Glycolysis is a sequence of reactions that catabolizes one molecule of glucose into two molecules of pyruvate with a net production of two ATP molecules. This process occurs anaerobically and takes place within the cytosol of cells. Pyruvate can further be metabolized anaerobically into lactate or through the TCA cycle to produce 39 ATP aerobically through the electron transport chain (Burton et al., 2004). The anaerobic process can be broken down into two stages. The first stage of glycolysis consists of

investing two molecules of ATP, whereas the second stage generates ATP through multiple reactions.

The first stage consists of glucose entry into the cell and its catabolism to glyceraldehyde 3-phosphate. Once glucose is transported into the cell it is immediately phosphorylated by a rate limiting step via hexokinase into glucose-6-phosphate (G6P) , which cannot pass through the glucose transporter (Whitesell et al., 2003). In hepatocytes, glucokinase is the enzyme that catalyzes the reaction of trapping glucose within the cell. This reaction destabilizes glucose by phosphorylating it which causes glucose to be more polar and the addition of negatively charged phosphate group prevents glucose from moving back through the membrane. Furthermore, the addition of the charged moiety to glucose destabilizing the structure and causes an increase in its energy, making it more reactive further facilitating the catabolism of glucose into glycolysis. During certain conditions, G6P can be synthesized into glycogen. For example, skeletal muscles are a major contributor to glucose uptake in the body and will incorporate glucose into glycogen (DeFronzo et al., 1981; Shulman et al., 1990). The next major step in stage one of anaerobic glycolysis is the irreversible reaction of phosphorylating fructose-6-phosphate into fructose 1,6-bisphosphate via phosphofructokinase (PFK) which is then cleaved into two molecules. These molecules set the stage for the second half of glycolysis.

The second stage of glycolysis generates ATP through the catabolism of two, 3 carbon units to produce pyruvate. The production of ATP occurs through two steps in glycolysis; 1) by the phosphoryl transfer from 1,3-bisphosphoglycerate via phosphoglycerate kinase and 2) and the phosphoryl transfer from phosphoenolpyruvate when converted to pyruvate via pyruvate kinase. Pyruvate, during anaerobic conditions

such as fasting or prolonged exercise, can be converted into lactate. Lactate can then be shuttled to the liver and recycled into glucose again. Additionally, pyruvate can enter the TCA cycle and be oxidized to produce $FADH_2$ and NADH which enter into the electron transport chain to produce energy, $CO_2$ and GTP/ATP molecules.

*Regulation of glycolysis*

Hexokinase. Hexokinase is the first rate-limiting step in skeletal muscle glycolysis and is inhibited by its product G6P. Low concentrations of G6P antagonize inhibition of the enzyme thus increasing its activity, while high concentrations of G6P inhibit the activity of hexokinase (White and Wilson, 1989). PFK activity also changes the enzyme activity of hexokinase. When PFK is inactive, the concentration of fructose 6-phosphate rises which in turn causes a rise in G6P causing a feedback inhibition on hexokinase(Lowry et al., 1964; Wilson, 2003).

Phosphofructokinase. Phosphofructokinase is the second rate-limiting step in glycolysis and the most important control of glycolysis in tissues. One major regulator of PFK in skeletal muscle is insulin. Increases in insulin postprandially will in turn cause enzyme activity of PFK to increase, impacting the rate of glycolysis in the cell (Ausina et al., 2018). It is well known that ATP and pH levels can regulate the activity of PFK in skeletal muscle. At high physiological values, ATP binds to PFK's low-affinity allosteric site causing a decrease in the enzyme's activity. This regulatory mechanism is more pronounced at pH lower than 7.2 which can be seen when the muscle is functioning anaerobically and producing excessive amounts of lactic acid (Lowry, 1965; Uyeda, 1979). When energy production falls, PFK activity is increased to meet the demands of the cell.

Pyruvate kinase. Pyruvate kinase catalyzes the third rate-limiting step in glycolysis and ultimately controls the flow out from this metabolic pathway. Along with PFK, pyruvate kinase can be regulated by the concentration of ATP. Increased ATP will cause a reduction in enzyme activity, ultimately slowing the pace of glycolysis (Holmsen and Storm, 1969). Finally, the concentrations of amino acids alanine and phenylalanine can also regulate the activity of pyruvate kinase. For example, phenylalanine inhibits the activity of pyruvate kinase by competition with the substrate phosphoenolpyruvate (PEP) in vitro (Schwark et al., 1971) and in vivo (Feksa et al., 2002). However, alanine prevents and reverses the inhibition that is caused by phenylalanine (Feksa et al., 2005).

*Pentose phosphate pathway*

The pentose phosphate pathway (PPP) oxidizes glycolytic intermediates to generate NADPH, a major reducing agent in cells. The PPP can also catabolize pentose sugars from the diet, synthesize pentose sugars for nucleotide biosynthesis and less commonly catabolize and synthesize four and seven carbon sugars. In most tissues 80-90% of glucose

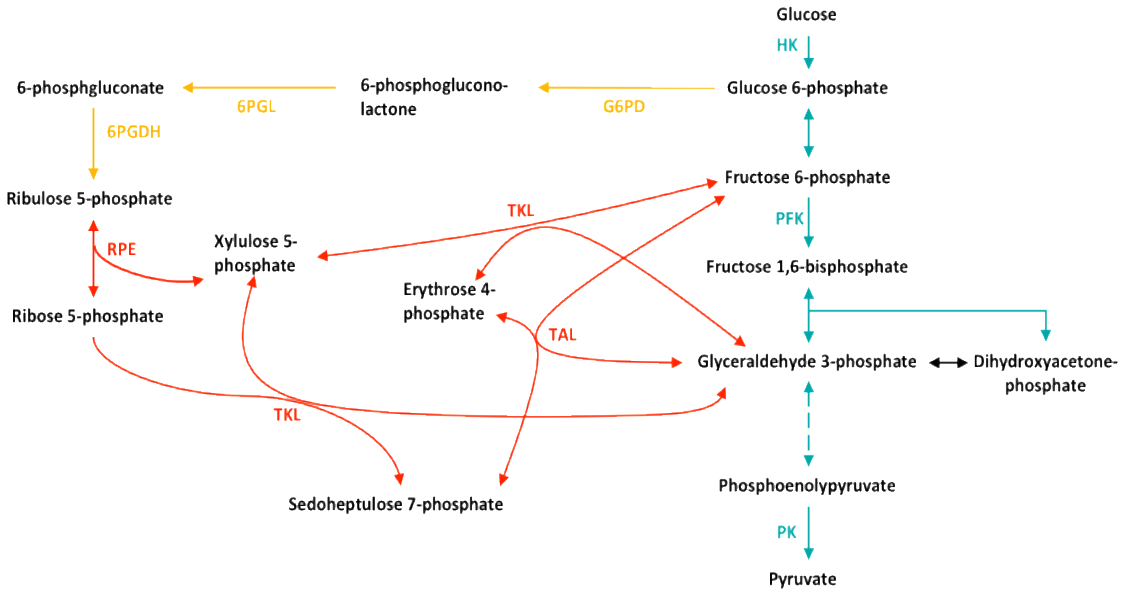


**Figure 1-2.** Pentose phosphate pathway. Pentose phosphate pathway branches from glucose 6-phosphate in the glycolysis (blue) and occurs in two phases; 1) oxidative phase represented in yellow and 2) non-oxidative phase represented in red (adatped from Stincone et al., 2015).

is oxidized through glycolysis while the remaining 10-20% occurs through the PPP (Wamelink et al., 2008). This pathway consists of two phases: 1) oxidative generation of NADPH and 2) the non-oxidative interconversion of sugars.

The oxidative phase of the PPP plays a major role in not only in supplying a major reductant (NADPH) for biosynthetic processes like fatty acid synthesis but also maintains the redox potential to protect against any oxidative stress in cells (Juhnke et al., 1996; Neuhaus and Emes, 2000). The oxidative branch consists of three irreversible reactions that produce NADPH using the enzymes glucose 6-phosphate dehydrogenase, gluconolactone and 6-phosphogluconate dehydrogenase. The end products of the oxidative phase are ribulose 5-phosphate, $CO_2$ and NADPH.

Unlike the oxidative phase of PPP, the non-oxidative phase is reversible. This branch of the pathway converts the five sugar carbon molecules into G6P and two other glycolytic intermediates, fructose 6-phosphate and glyceraldehyde 3-phosphate. The ribulose 5-phosphate that is produced from the oxidative phase can be isomerized into products used for nucleotide and nucleic acid synthesis.

*Serine biosynthetic pathway*

The serine biosynthetic pathway is another pathway that utilizes the glycolytic intermediate 3-phosphoglycerate (3-PG). Synthesis of serine begins with the first enzyme 3-phosphoglycerate dehydrogenase (PHGDH) (Snell, 1985). PHGDH utilizes NAD to oxidize 3-PG into phosphohydroxypyruvate (Achouri et al., 1997). Through transamination and phosphate ester hydrolysis, phosphoserine aminotransferase 1 (PSAT1) and phosphoserine phosphatase produce serine. Serine is essential for the synthesis of proteins and for molecules that are utilized in cell proliferation and produces an important

intermediate of the one carbon metabolism pathway, methylenetetrahydrofolate (Daly and Aprison, 1974; Snell and Weber, 1986). In addition, the serine biosynthetic pathway is increased by the up-regulation of PHGDH and serine hydroxymethyl transferases (SHMT) when cell proliferation is in high demand (Snell and Weber, 1986; Snell et al., 1987; Snell et al., 1988).

*Tricarboxylic acid cycle and electron transport chain*

The anaerobic process of catabolizing glucose to pyruvate via glycolysis only produces a small fraction of ATP that is available from glucose. A majority of the ATP that is generated in metabolism is through an aerobic process. The TCA cycle through the oxidation of pyruvate, can generate molecules of $FADH_2$ and NADH which enter into the electron transport chain. In addition, the TCA cycle is involved in both a anaplerotic and cataplerotic biochemical role (Owen et al., 2002). Anaplerotic sequences was a term first used to describe reactions or pathways that replenish the pools of intermediate metabolites of the TCA cycle (Kornberg, 1966). TCA cycle intermediates are important for the functioning of the TCA cycle such as the oxidation of acetyl-CoA to carbon dioxide. The pool size of TCA cycle intermediates do not vary over a large range of metabolic changes such as high energy consumption (exercise) or during low energy consumption such as fasting (Graham and Gibala, 1998). Anaplerosis of TCA intermediates must also be balanced with cataplerosis in order to balance the efflux and influx of carbons (Owen et al., 2002). Cataplerosis in the TCA cycle is defined as the reactions involved in the disposal of intermediate metabolites. This biochemical role can be linked to pathways such as GNG in the liver and the cortex of the kidney and fatty acid synthesis in the liver (Owen et al., 1969; Jungas et al., 1992; Hakimi et al., 2005). The TCA cycle is a delicate balance

between the input and output of intermediates for various metabolic processes and is regulated by key steps. The TCA cycle is regulated by acetyl CoA and its intermediates. For example, acetyl CoA can be derived from many sources including the oxidation of glucose through glycolysis and the oxidation of fatty acids as well as the breakdown of certain amino acids, thereby making the substrate availability a regulator of the production of acetyl CoA (Abdel-aleem et al., 1994; Abdel-aleem et al., 1996). Another regulator of the TCA cycle is citrate synthase (CS). Citrate synthase controls the metabolic flux into the TCA cycle from acetyl CoA. If acetyl CoA is decreased then the activity of CS will be reduced along with reduced concentrations of oxaloacetate. In addition citrate synthase is also inhibited by NADH and succinyl-CoA (Wiegand and Remington, 1986). The TCA cycle is key to unifying other metabolic pathways together including carbohydrates, lipids and proteins.

**Fatty Acid Metabolism**

The digestion of dietary lipids begins in the stomach by lingual lipase and continues in the small intestine with gastric and pancreatic lipases. These lipases show a higher activity for short- and medium-chain fatty acids (MCFA) than for long-chain fatty acids (LCFA), which produce free fatty acids and 2-monoglyceride and results in dietary medium-chain triglycerides and long-chain triglycerides having differing metabolic pathways in digestions and absorption (Bloom et al., 1951; Bach and Babayan, 1982; Carey et al., 1983). Medium-chain triglycerides are degraded to be absorbed either as 3 fatty acids and the glycerol backbone or absorbed intact and absorbed from the small intestinal cells into the portal vein to be transported to the liver (Bach and Babayan, 1982; Carey et al., 1983). In contrast, LCFA are usually emulsified in the small intestine with bile salts

and are then able to cross into the enterocyte where they are formed back into triglycerides and incorporated into chylomicrons that enter the lymphatic system (You et al., 2008). Fatty acids play a major role in metabolism in that they are highly efficient fuel molecules that are stored as triglycerides in adipose tissue. For example, long chain fatty acid palmitate when completely oxidized produces 106 ATP (Berg et al., 2002b).

During fasting lipid oxidation becomes the predominant fuel in the resting skeletal muscle (Kim et al., 2000). Free fatty acids are mobilized and oxidized to produce ATP through β-oxidation in the mitochondria. Long chain fatty acids are transported across the outer mitochondria membrane in the form of acyl-CoA and is a rate limiting step of β-oxidation because of the dependency of the enzyme carnitine palmitoyltransferase (CPT-1) to shuttle it across the mitochondria membrane as acylcarnitine (Dagher et al., 2001; Bruce et al., 2007). Decreases in CPT1 activity results in lipid accumulation and insulin resistance in rats placed on a high fat diet (Dobbins et al., 2001). However, overexpression of skeletal muscle CPT1 ameliorated the effects of insulin resistance induced by high fat feeding (Bruce et al., 2009). Once in the mitochondrial matrix, acyl-CoA is shortened by two carbons producing acetyl CoA and one molecule of NADH and $FADH_2$ (Lopaschuk et al., 2010). The resulting acetyl-CoA enters into the TCA cycle to undergo further oxidation to produce NADH and $FADH_2$, whereas the NADH and $FADH_2$ generated from β-oxidation directly are utilized by the electron transport chain to generate ATP molecules.

**Table 1-1.** Essential and nonessential amino acids in mammals

| Essential Amino Acids | Nonessential Amino Acids |
|---|---|
| Arginine[1] | Alanine |
| Histidine | Asparagine |
| Isoleucine | Aspartate |
| Leucine | Cysteine[2] |
| Lysine | Glutamate |
| Methionine | Glutamine[2] |
| Phenylalanine | Glycine |
| Threonine | Proline[3] |
| Tryptophan | Serine |
| Valine | Taurine[4] |
| | Tyrosine |

[1] Arginine is essential for young animals although it may not be required in the diet in adult species (humans, pigs and rats).
[2] Conditionally essential AA in neonates
[3] Essential for young pigs
[4] Essential for carnivores (cats)

**Amino acid metabolism**

Dietary protein digestion begins at the stomach, where the acid environment promotes the denaturing process, making proteins more accessible to proteolysis. Protein digestion continues in the small intestine where they are degraded to amino acids and oligopeptides and are absorbed across the intestinal wall and released into the bloodstream where they are absorbed by tissues. The primary use for amino acids is as a building block for protein synthesis and other nitrogenous compounds such as nucleotide bases (Moffatt and Ashihara, 2002; Lecker et al., 2006).

Adequate supply of dietary amino acids is essential for growth, development, survival and overall health of animals and humans (Wu, 2009, 2010; Ren et al., 2012). Amino acids are classified as nutritionally indispensable (essential) and dispensable (non-essential). Nutritionally indispensable amino acids are defined by those whose whole

carbon skeletons cannot be synthesized de novo in the body or those that are inadequately synthesized de novo by the body in relation to the cellular needs such as growth and development. These amino acids must be provided by the diet in order to meet requirements. Dispensable amino acid can be defined as those that are synthesized in the body in adequate amounts to meet requirements, without dietary supplement. In addition, there are amino acids that are conditionally essential which consist of amino acids that are normally synthesized de novo in adequate amounts however during conditions where rates of utilization exceed that of synthesis these amino acids must be provided in the diet (Wu, 2009). Amino acids also play a major role in energy metabolism which will be discussed in more detail.

**Gluconeogenesis**

Gluconeogenesis (GNG) is defined as synthesizing new glucose from non-carbohydrate sources, like amino acids and glycerol. GNG occurs mainly in the liver with small amounts in the cortex of the kidney. Skeletal muscle does not have the ability to produce glucose due to lacking the enzyme glucose-6-phosphatase (Rajas et al., 1999). In glycolysis, glucose is converted to pyruvate and in GNG pyruvate is converted to glucose. Although it seems like they are reciprocal of one another, there are major differences between the two pathways.

The three rate limiting reactions in glycolysis; hexokinase, PFK and pyruvate kinase are energetically favorable in the forward reaction, however in reverse these reactions require more energy in order to overcome these high exergonic steps. In order for GNG to produce glucose, it requires energetically favorable steps in order to bypass the rate limiting steps of glycolysis. The first is converting pyruvate to oxaloacetate via

pyruvate carboxylase. Through cataplerosis, oxaloacetate is converted to phosphoenolpyruvate using phosphoenolpyruvate carboxykinase (PEPCK). There are two distinct forms of PEPCK, a mitochondrial (PEPCKM) and a cytosolic (PEPCKC). The role of PEPCKC has extensively been reviewed in hepatic and renal GNG (Hanson and Patel, 1994). Deletion of the PEPCKC gene in the liver leads to shut down of GNG (She et al., 2003; Hakimi et al., 2005). In addition, total body deletion of the gene results in hypoglycemia and ultimately death, indicating that PEPCKC plays a major regulatory role on the metabolic pathway. An increase in PEPCK in the liver of rats fed a low protein diet exhibited increased expression of serine biosynthetic enzymes (Fallon et al., 1966). The first prominent role PEPCKM was identified for the mechanism of glucose-stimulated insulin secretion through the synthesis of PEP in the liver (Stark et al., 2009). In addition, the silencing of PEPCKM in the liver lowers plasma glucose, insulin and triglycerides, along with depleting glycogen stores and reducing white adipose tissue (Stark et al., 2014). In the skeletal muscle of pigs fed a β-adrenergic agonist, PEPCKM was increased along with the increase in the serine biosynthetic pathway enzymes indicating an anaplerotic effect on the synthesis of serine (Brown et al., 2016). Together, this indicates that PEPCK not only plays a role in GNG but in possibly provided carbons from the TCA cycle to the serine biosynthesis pathway when needed. The second rate-limiting step of converting fructose 1,6-bisphosphate to fructose 6-phosphate is catalyzed by fructose 1,6-

bisphosphatase. The final step rate-limiting step is the conversion of glucose 6- phosphate to glucose catalyzed by glucose 6-phosphatase.

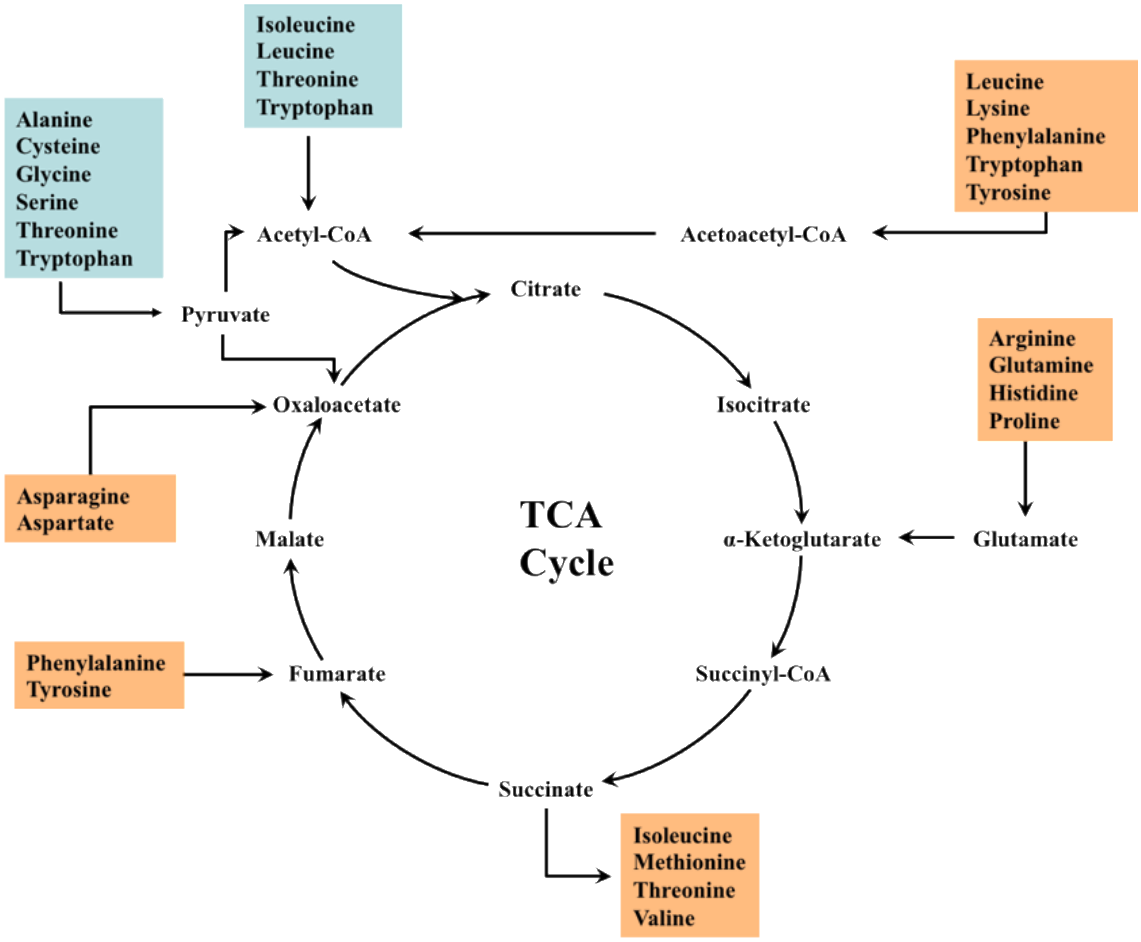


**Figure 1-3**. Glucogenic and ketogenic amino acids. Ketogenic amino acids are catabolized to acetyl-CoA. Glucogenic amino acids are catabolized to pyruvate. Blue represent ketogenic amino acids and orange represent glucogenic amino acids (adapted from Berg et al., 2002a).

Amino acids. Catabolism of amino acids accounts for up to 10-15% of energy production (Chang and Goldberg, 1978). The major site for the use of amino acids for metabolic fuel is in the liver and cortex of the kidney, although skeletal muscle readily degrade branch chain amino acids (valine, leucine and isoleucine) (Chang and Goldberg, 1978). Amino acid breakdown during metabolism can be described in two phases: 1) the removal of the α-amino group and 2) the carbon skeleton is converted into major metabolic pathway intermediates (D'Andrea, 2000). In the first phase the α-amino group of many

amino acids is transferred to α-ketoglutarate to form glutamate, which can then be deaminated to form ammonium ion. The ammonium ion is then converted into urea, however some of the ammonium ions formed in the breakdown of amino acids is recycled in the biosynthesis of nitrogen compounds. The second phase is dedicated to ultimately form intermediates that produce glucose, fat or oxidized by the TCA cycle. Amino acids can further be characterized as ketogenic or glucogenic. Ketogenic amino acids are those that their carbon skeletons are broken down directly or indirectly to acetyl-CoA which also give rise to ketone bodies which can be converted to fatty acids. Glucogenic amino acids are characterized as those that are degraded to intermediates such as pyruvate, α-ketoglutarate, fumarate, etc. that increase the net synthesis of glucose (D'Andrea, 2000). Amino acids such as phenylalanine, tyrosine, threonine and isoleucine can be characterized as both glucogenic and ketogenic amino acids due to the multiple pathways or degradation. Altogether, amino acids play a large role in growth and development and as a crucial metabolic fuel.

Glycerol. During prolonged fasting when glycogen stores are low, lipids are mobilized to produce energy. Triglycerides are hydrolyzed through lipolysis. Lipolysis of triglycerides releases free fatty acids and glycerol. The contribution of free fatty acids to glucose production remains unclear. In some studies, the direct relationship between plasma free fatty acids and fasting glucose production rates was found (Ferrannini et al., 1983; Boden and Jadali, 1991; Rebrin et al., 1996). However, in other studies no significant effects of increased free fatty acid levels on glucose production were found (Roden et al., 2000; Chu et al., 2002). Glycerol, however, can be converted into glucose by ultimately being converted to dihydroxyacetone phosphate and entering into GNG at glyceraldehyde

3-phosphate. The contribution of glycerol to the production of glucose in healthy individuals during fasting is ~36%, making it a large contributor to glucose production in the body (Baba et al., 1995).

**Metabolic Flexibility**

Metabolic flexibility was first described in parasitic worms as the increased capacity to generate energy and key metabolites using anaerobic or aerobic respiration in order to adapt to environmental changes (Kohler, 1985). The Randle Cycle was a foundation to explain the elevated fatty acid oxidation coupled with reduced glucose oxidation in response to insulin resistance and type 2 diabetes (Randle et al., 1963). The more recent concept is the pertaining to fuel selection during the transition from fasting and fed states or fasting to insulin stimulation in order to explain insulin resistance (Storlien et al., 2004). In addition, metabolic flexibility encompasses more metabolic circumstances, such as nocturnal and diurnal conditions (Kelley et al., 1999) or exercise training (Rasmussen et al., 2014).

*Fasting and fed flexibility*

During the transition from fasting to feeding there is a shift in fuel selection from primarily fatty acid oxidation to glucose oxidation in skeletal muscle (Andres et al., 1956). Furthermore, this shift also includes a small increase in glycolytic energy production to efficiently utilize the varying mixture of macronutrients in the diet (Kelley et al., 1999). The primary purpose of the shift in metabolic fuel selection during fasting and fed states is to move from catabolic and anabolic processes to store energy in tissues (Goodpaster and Sparks, 2017).

Maintaining metabolic homeostasis during fasting and feeding relies on the ability of the tissue or organ to control the available fuel on a systemic level. For example, after a carbohydrate-rich meal pancreatic β-cells respond by releasing insulin into the bloodstream and in turn increasing the insulin to glucagon ratio (Smith et al., 2018). Insulin influences glucose uptake and absorption into multiple tissues. In the liver, insulin stimulates glycogen synthesis in turn decreasing glycogenolysis and GNG (Aiston et al., 2003). Skeletal muscle plays a major role in insulin-stimulated glucose clearance when insulin binds to its receptor and activates the translocation of GLUT4 to the plasma membrane (Dimitriadis et al., 2011). The rise in glycolysis and pyruvate concentrations from glycolysis suppress the inhibition of pyruvate dehydrogenase kinases (PDK) on pyruvate dehydrogenase complex (PDC), in turn reducing the phosphorylation of pyruvate dehydrogenases (PDH) increasing glucose oxidation (Zhang et al., 2014). In addition, the increase in glucose uptake, glycolysis and pyruvate oxidation causes a decrease in malonyl-CoA, which inhibits CPT-1 (Muoio, 2014). The inhibition of CPT-1 and increase in insulin is also coupled with a response from adipose tissue to decrease lipolysis and increase fatty acid and triglyceride synthesis (Dimitriadis et al., 2011). Altogether, this systemic response postprandial ensures minimal exposure of the tissues to hyperglycemia and that any nutrients in excess are stored to be released in times of metabolic need or scarcity.

During fasting, circulating carbohydrates and lipids decrease along with the insulin to glucagon ratio causing a switch from primarily glucose oxidation to fatty acid oxidation (Longo and Mattson, 2014). Glucagon mobilizes glycogen in response to low glucose levels through hepatic glycogenolysis (Parrilla et al., 1974) and stimulates ketogenesis (McGarry et al., 1975). In addition, the decrease in insulin suppressing the synthesis

hepatic malonyl-CoA and lipogenesis is coupled with the activation of fatty acid oxidation (McGarry and Foster, 1980; Ramnanan et al., 2011). This is mediated by the activation of adenosine monophosphate-activated protein (AMPK) inhibiting acetyl-CoA carboxylase (ACC). Inhibiting ACC results in decrease malonyl-CoA concentrations in turn increasing CPT-1 activity. The increase in CPT-1 activity will ultimately increase the transport of some fatty acids into the mitochondria to undergo β-oxidation (Gray and Kim, 2011). During an increase in fatty acid oxidation, NADH and acetyl-CoA concentrations rise causing an inhibition on PDH activity by activating PDK (Zhang et al., 2014). In addition, the increase in fatty acid oxidation increases the activation of PDK gene expression via fatty acid dependent peroxisome proliferator-activated receptor (PPAR) signaling (Sugden and Holness, 2006). Increases in the metabolite citrate is also involved in the switch from glucose oxidation during fed to fatty acid oxidation during fasting. For example, the increase in fatty acid oxidation increases citrate. The increase in citrate has feedback inhibition on PFK, decreasing the second rate-limiting step in glycolysis. The inhibition of this step causes an increase in glucose 6-phosphate concentrations, which has feedback inhibition on hexokinase further decreasing the catabolism of glucose through glycolysis (Spriet, 2014). These changes favor the oxidation of fatty acids in order to conserve glucose and in addition utilize GNG substrates to increase glucose production in order to minimize hypoglycemia during times of fasting.

**Metabolic Inflexibility**

In terms of respiration quotient (RQ), which is the ratio of carbon dioxide production to oxygen consumption, metabolic flexibility goes from a low RQ during fasting to a high RQ during feeding (Kelley et al., 1999). Metabolic inflexibility is present

in individuals who are insulin resistant and their RQ during fasting and fed states are unchanged, meaning they continue to oxidize a fixed mixture of fatty acids and glucose regardless of the nutritional content (Kelley et al., 1999). Thus in the context of over-feeding, the control of tissues over fuel availability is lost and competition among them rise leaving the mitochondria in a state of indecision leading to continuous oxidation of all three major substrates (Muoio, 2014).

*Skeletal muscle and insulin resistance*

Insulin resistance is characterized by an increase in intramuscular triglyceride accumulation and in lean and obese individuals is inversely related to insulin sensitivity (Goodpaster et al., 1997; Pan et al., 1997; Greco et al., 2002). In addition, plasma free fatty acid levels were increased through a lipid and heparin infusion and resulted in increased intramyocellular triglyceride content and insulin resistance, indicating that intramuscular triglycerides play a metabolic role in metabolic diseases (Boden et al., 2001).

As stated above, skeletal muscle in healthy lean individuals can switch fuels during fasting and fed states efficiently. In obese and individuals of type 2 diabetes, metabolic flexibility is impaired in skeletal muscle (Kelley and Mandarino, 1990). Oxidation of fatty acids in the skeletal muscle of obese or type II diabetic individuals during fasting conditions does not increase, instead glucose oxidation was elevated (Kelley and Mandarino, 1990; Kelley et al., 1999). In addition, during fed conditions these individuals did not exhibit a sharp increase in glucose oxidation as seen in normal healthy subjects instead it was blunted. The inability to control the metabolic switch and utilize fatty acids as efficiently in obese individuals may be the underlying mechanism to accumulation of intramuscular triglycerides in skeletal muscle (Kelley and Mandarino, 2000).

In summary, the regulation of glucose homeostasis in LBWT neonatal pigs remains unclear. Given that glycerol accounts for a majority glucose production during fasting, future studies are required to investigate nutritional manipulations for increasing glucose production in LBWT neonatal pigs. Moreover, future studies are required to investigate the changes in glucose catabolism in the skeletal muscle of LBWT neonatal pigs.

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# Chapter 2 Lipid Intake Does Not Influence Glucose Homeostasis in Three-Week Old Low Birth Weight Neonatal Pigs

**Abstract**

Previous data in our lab suggests that despite feeding comparable amounts of the same diet, low birthweight (LBWT) neonatal pigs exhibit lower weight gain, fat and protein deposition than their normal birthweight (NBWT) littermates. The objective of this study was to investigate growth performance and how dietary energy intakes affect growth and glucose kinetics in LBWT neonatal piglets. Day old piglets ($n$=6) were fed isonitrogenous diets providing of either 80% (LE) or 100% (HE) of metabolizable energy requirements. Body composition was assessed using dual energy x-ray absorptiometry at day -1 and 13 of the study. On days 0 and 13 of the study a fasting intravenous glucose tolerance test (IVGTT) was administered. On day 14 of the study, glucose and glycerol kinetics were assessed using stable isotope tracer kinetics. At the termination of the study muscles and visceral organs were weighed. Increasing dietary energy did not affect body weight of LBWT or NBWT pigs, and LBWT pigs weighed less than their NBWT siblings ($P$<0.05). Liver and *longissimus dorsi* weights as a percentage of whole-body weight was greater for piglets fed HE diet compared with those fed LE diet ($P$<0.05). Although glucose concentration was modestly lower in LBWT compared to NBWT group ($P$<0.05), there were no differences in glucose clearance or appearance across all groups. The contribution of glycerol to gluconeogenesis was similar across all groups. Our data suggest that lipid supplementation modestly improved growth of skeletal muscle and the liver, however, hepatic fat content increased in LBWT and NBWT pigs. In addition, glucose concentration remained lower in LBWT pigs and lipid supplementation had no effect on glucose homeostasis in LBWT and NBWT pigs. These data suggest that the hyperglycemic effect of lipid supplementation may depend on the route of administration and/or age of the

neonate. In conclusion, lipid supplementation modestly improved growth of skeletal muscle and liver at the expense of increased fat content in the liver. In addition, lipid supplementation had no effect on glucose homeostasis in LBWT and NBWT pigs suggesting that the hyperglycemic response is dependent on administration route and age of neonate.

**Keywords:** low birth weight, growth, glucose metabolism, fat intake

## Introduction

Intrauterine growth restriction which results from impaired growth and development of the mammalian fetus or its organs during pregnancy, is a major concern in perinatal medicine (McMillen and Robinson, 2005) and animal production (Wu et al., 2006). Intrauterine growth restriction is one of the leading causes that contribute to low birth weight (LBWT) in infants, i.e. having a birthweight below the $10^{th}$ percentile (Rosenberg, 2008b), and affects 10-15% of all births in developing countries (Berghella, 2007; Saleem et al., 2011). In addition to decreasing the chances of survival, and the permanent stunting effect, LBWT infants are at a higher risk of perinatal morbidity and mortality along with life-long metabolic diseases, including insulin resistance, type II diabetes, cardiovascular disease and obesity (Barker, 2000; Godfrey and Barker, 2000; Resnik, 2002). The neonatal pig is a good surrogate for studying human infants due to similarities in growth and metabolism and the natural prevalence of LBWT (Quiniou et al., 2002; Wu et al., 2006). In addition, the existence of LBWT and NBWT pigs in the same litter reduces genetic variation and parental influence.

Before birth, glucose levels are maintained by trans-placental transfer of glucose from the mother, however there is a critical period between birth and the establishment of suckling when the neonate has to rely on glycogenolysis to maintain blood glucose levels (Hillman et al., 2012). Due to limited glycogen stores, gluconeogenesis (GNG) inevitably becomes the main source of glucose production from glucogenic substrates like amino acids and glycerol. After birth, up to 50% of LBWT infants have transient hypoglycemia with elevated plasma lactate and amino acid concentrations (Haymond et al., 1974; Sunehag and Haymond, 2002). Despite the elevated concentrations of these substrates,

GNG from alanine is similar in LBWT and normal birth weight (NBWT) infants postnatally (Frazer et al., 1981). Conversely, glycerol accounts for up to 65% of total GNG, indicating that glycerol is a major substrate for hepatic glucose production in LBWT infants (Collins et al., 1992; Sunehag et al., 1999). In support of this view, feeding high dietary fat improves plasma glucose concentration in newborn infants (Sann et al., 1982; Sann et al., 1988a; Bougneres et al., 1989) and pigs (Le Dividich et al., 1991). Since LBWT neonates have low body fat content, we hypothesized that increasing energy intake through dietary lipid supplementation will improve muscle growth and glucose production in LBWT neonatal pigs. Thus, the objectives of this study were to: 1) investigate the changes in growth performance and body composition, and 2) determine changes in glucose and glycerol kinetics, when dietary lipid supplies are increased in LBWT piglets.

**Materials and Methods**

*Animals and Surgeries.* Gestating sows were fed a corn-soybean based diet to meet NRC requirements (Committe of Nutrient Requirements of Swine, 2012) with free access to water. At birth, piglets were weighed and characterized as normal (NBWT) or low (LBWT) birth-weight when their body weight was within ±0.5 SD or ≤2 SD of the litter mean (D'Inca et al., 2010). Twelve 3-d old NBWT pigs were matched to twelve LBWT same-sex siblings. After an overnight fast, pigs were fitted with indwelling Tygon™ (AAQ0427, Tygon™ Company) catheters placed in the carotid artery and jugular vein using sterile techniques under general isoflurane anesthesia. Pigs were allowed to recover from surgery for 3 days and fed a sow milk replacer. All animal procedures were approved by Virginia Tech Institutional Animal Care and Use Committee.

*Diets.* NBWT and LBWT piglets were fed a low energy (LE) or a high energy (HE) milk replacers (Table 1). The increase in energy intake was done by increasing the fat content of the diet keeping all other nutrients constant. Piglets were fed at 240 ml • $kg^{-1}$ •$d^{-1}$ for 14 days, and weighed every other day and intake adjusted accordingly.

*Dual Energy X-ray Absorptiometry.* Body composition was determined by dual energy X-ray absorptiometry (DXA, GE Healthcare, USA) on day -1 and 13 of the study. A full body scan was done under light isoflurane sedation for the duration of the scan. Pigs were fasted overnight before each scan to reduce the effect of gut fill on body composition.

*Fat extraction.* Frozen liver samples were weighed out (200 mg) and homogenized using 6850 Freezer/Mill® by Spex Sample Prep (Metuchen, NJ). Homogenized samples were weighed on filter paper and lyophilized. Dried samples were weighed again and placed in a soxhlet extractor. Lipid was extracted using hexane for 24 hrs. Samples were then kept in a desiccator and allowed to dry overnight to be weighed.

*Intravenous glucose tolerance test.* Glucose clearance was measured by an intravenous glucose tolerance test (IVGTT) on day 0 and 14 of study. A bolus dose of glucose was given (1 g • $kg^{-1}$) within one minute and blood samples taken at 0, 10, 20, 30 and 60 minutes following the dose (Stoll et al., 2012). Plasma was separated from heparinized blood by centrifuging at 4°C for 10 min and 3200 *g*.

*Infusion and sampling.* [6,6-$^{2}H_2$]-glucose, [U-$^{13}C_6$]-glucose, [2-$^{13}$C]-glycerol, and [3-$^{13}C_3$]-glycerol were purchased from Cambridge Isotopes Laboratories (Andover, MA). On day 15 following the initiation of feeding, piglets were fasted overnight and a sterile solution containing [6,6-$^{2}H_2$]-glucose (2.64 μmol • $kg^{-1}$• $min^{-1}$) and [2-$^{13}$C]-glycerol (0.744 μmol • $kg^{-1}$ • $min^{-1}$) was infused into the jugular vein catheter for 8 h. Blood samples were

taken every hour throughout the infusion period. By hour 4, piglets were fed a meal representing 1/5$^{th}$ of their respective daily dietary allowance, and plasma collected every 60 min as described above. After 4 h piglets were fed another meal and euthanized 60 min later. Muscle and organ samples were weighed immediately after euthanasia.

*Glucose and glycerol concentration and enrichment.* To a known amount of fresh plasma was added an equal amount of a solution containing [U-$^{13}C_6$]-glucose or [3-$^{13}C_3$]-glycerol internal standards. Glucose was analyzed by gas chromatography-mass spectrometry (GC-MS) as previously described (Hannestad and Lundblad, 1997). Briefly, the aldonitrile pentaacetate derivative was formed and ions at *m/z* 314/319 ([U-$^{13}C_6$]-glucose), *m/z* 217/219 ([6,6-$^{2}H_2$]-glucose), and *m/z* 212/213 ([2-$^{13}$C]-glycerol incorporated into glucose) were monitored in electron ionization mode (Hannestad and Lundblad, 1997). Glycerol was converted to its t-butyldimethylsilyl derivative (Flakoll et al., 2000) and ions at *m/z* 377/378 *(*[2-$^{13}$C]-glycerol) and *m/*z 377/380 ([3-$^{13}C_3$]-glycerol) were monitored in electron ionization mode. Standard curves were generated and spillover corrections were made for tracers and internal standard.

*Calculations.* When steady state was achieved prior to a meal, glucose and glycerol rate of appearance $Ra$ (mmol•kg$^{-1}$•h$^{-1}$) was calculated using the following equation:

$$Ra = \left(\frac{E_t}{E_A} - 1\right) \times IR$$

where $E_t$is the enrichment of the glucose or glycerol tracer infused, $E_A$ is the arterial enrichment of glucose or glycerol, and $IR$ is infusion rate (mmol•kg$^{-1}$•hr$^{-1}$) of either [6,6-$^{2}H_2$]-glucose or [2-$^{13}$C]-glycerol tracers. Following a meal, $Ra$ was calculated according to a modified Steele's equation (Proietto et al., 1987) for non-steady state conditions:

$$Ra = \frac{\left(IR - \left(p \times C_m(t) \times \frac{dE}{dt}\right)\right)}{E_m(t)}$$

where $IR$ is infusion rate (mmol • $kg^{-1}$ • $h^{-1}$) of either [6,6-$_2H^2$]-glucose or [2-$^{13}$C]-glycerol tracers, $p$ is the pool fraction for instant mixing (glucose, $p$ = 0.65; glycerol, $p = 0.63$), $V_d$ is volume of distribution (glucose, $V_d = 0.2$ ; glycerol, $V_d = 0.4$), $C_m(t)$ is the mean glucose or glycerol arterial concentration (mmol • $l^{-1}$), $\frac{dE}{dt}$ is the change in glucose or glycerol enrichment between successive time points, and $E_m(t)$ is the mean enrichment of glucose or glycerol between two time points. Gluconeogenesis (GNG) from glycerol (mmol • $kg^{-1}$ • $h^{-1}$) was calculated using the following equations (Peroni et al., 1995):

$$\%Glucose\ from\ Glycerol = \left(\frac{E_{glucose}}{\left(2 \times E_{glycerol}\right)}\right) \times 100$$

$$GNG\ from\ Glycerol = \%glucose\ \times\ glucose\ Ra$$

where $E_{glucose}$ is the $^{13}$C glucose and $E_{glycerol}$ is $^{13}$C glycerol isotopic enrichments.

*Insulin concentrations.* Plasma insulin concentrations were measured before and after a meal using Porcine Insulin ELISA Kit (R&D systems).

*Statistical analysis*. Data were analyzed by PROC MIXED procedure in SAS version 9.3 (SAS Inst. Inc., Cary, NC). For comparisons of the measurements in plasma, body weight, muscle and organ weights between LBWT and NBWT neonatal pigs, sow and sex were the random effects. When a significant treatment effect was detected, means were compared using Tukey-Kramer Multiple Comparison Test. Data are expressed as the least squares means ± SEM and differences considered significance at $P < 0.05$, unless otherwise noted.

**Results**

*Growth Performance.* Bodyweight was consistently greater in NBWT than LBWT piglets ($P$ <0.01), however, fractional growth rates among all groups did not differ, indicating that growth was proportional to birth weight and was not affected by diet (Figure 1A and 1B). Increasing energy in the diet did not change feed conversion, nor did the deposition energy and protein in NBWT and LBWT piglets (Figure 2A-C). Fat mass increased over time for NBWT and LBWT piglets ($P$ <0.01) with NBWT piglets having a higher fat content than LBWT piglets, regardless of diet ($P$ <0.01, Figure 3A). Similarly, percent body fat increased between the beginning and the end of the feeding period ($P$ <0.01), and was higher in NBWT piglets than LBWT piglets, regardless of diet ($P$ <0.01, Figure 3B). Lean mass increased from day 0 to day 14 in all piglets ($P$ <0.01), and lean mass was lower in LBWT compared to NBWT littermates (Figure 3C). However, lean mass as percent of body weight was similar at day 0 and day 14 for all piglets except for LBWT HE where lean mass was lower by the end of the study ($P$ <0.05, Figure 3D). Bone mineral content was higher in NBWT than LBWT pigs and maintained throughout the study ($P$ <0.05, Figure 3E). Bone mineral content as a percentage of body weight did not differ among piglets, however it decreased during the study ($P$ <0.01, Figure 3F).

*Organ and Muscle*. Although heart, liver and kidney were consistently heavier in NBWT compared to LBWT pigs ($P$ <0.05), when expressed as a percent of body weight only liver represented a 6% lower proportion of body weight in NBWT compared to their LBWT siblings ($P$ <0.05, Figure 4). In addition, increasing energy intake increased the fractional weight of the liver by 26% ($P$ <0.01) in pigs consuming HE compared to those on the LE diet. LD, soleus and gastrocnemius were consistently heavier in NBWT

compared to LBWT pigs ($P$ <0.01, Figure 5), however when expressed as a percent of body weight only LD represented a larger proportion in NBWT compared to LBWT pigs ($P$ <0.05). Furthermore, HE diet fed piglets had a 21% increase in fractional weight of the LD ($P$ <0.05) compared to those fed a LE diet (Figure 5B).

*IVGTT and Glucose Kinetics*. Glucose clearance measured at day 0 and 14 was not different between NBWT and LBWT piglets, regardless of diet or growth status (Figure 6A and B). Plasma glucose concentration, measured during infusion of [6,6-$^{2}H_{2}$]-glucose on day 15 of the study over a four-hour period post-feeding (Figure 7A), was on average 9% lower in LBWT than in NBWT groups, regardless of diet fed ($P$<0.05). Plasma glucose appearance rate increased post-meal ($P$ <0.01) but did not differ among NBWT and LBWT piglets, regardless of diet (Figure 8B)

*Glycerol Kinetics.* Plasma glycerol concentration measured during the last four hours of [2-$^{13}$C]-glycerol infusion on day 15 of the study (Figure 7C) was similar for all piglets. Glycerol rate of appearance was similar in piglets fed HE over time, however, LBWT LE fed piglets were higher at hour 2 and 3 and lower by hour 4 in comparison to NBWT LE fed piglets ($P$ <0.01, Figure 7D). The contribution of glycerol to glucose decreased in the first hour immediately following a meal and increased for NBWT pigs fed the LE diet ($P$ <0.01) between 3 and 4 h post feeding (Figure 7E). This pattern was similar to the contribution of glycerol to GNG which increased between 3 and 4h ($P$ <0.01, Figure 6).

**Discussion**

Although the majority (~75-95%) of LBWT infants undergo a period of catch-up growth by 2 years of age, (Soto et al., 2003; Beger et al., 2018), they are at risk of developing metabolic disorders (Soto et al., 2003). It is not known whether these metabolic disturbances start immediately in the postnatal period. We set out to determine whether increasing energy supplementation in the early postnatal period to enhance growth would affect glucose homeostasis in LBWT neonatal pigs.

In neonatal pigs, a reduction in feed intake is expected when dietary fat concentration increases (Le Dividich et al., 1991). Thus, to control for variations in feed intake, and to ensure the consumption of the offered formulas, pigs were limit-fed the same amounts of LE or HE formulas per unit of body weight. Our results indicated that body weight was less in LBWT compared to NBWT pigs and was comparable irrespective of energy supplementation. Previous studies indicate that pigs go through a period of catch-up growth between 3 and 12 months of age when fed *ad libitum* (Poore and Fowden, 2002; Myrie et al., 2011). Obviously, in the current study, pigs were fed the experimental diets for only 14 d which may have precluded detecting small differences in body weights. This is why muscle weights was determined. Although longissimus muscle mass was lower in LBWT compared to NBWT pigs, longissimus muscle mass as a proportion of body weight was enhanced by energy supplementation and remained less in LBWT pigs compared to their NBWT siblings. While LBWT pigs may reach the same weight as their littermates, they often accrete less lean tissue and more fat (Poore and Fowden, 2004; Gondret et al., 2006). In this connection, it is well established that poor fetal nutrition is not only associated with low birthweight, but also results in unequal organ growth postnatally (Wells, 2011). This asymmetrical growth pattern occurs virtually in all mammals including LBWT infants (Kilavuz

and Vetter, 1999; Damodaram et al., 2012), pigs (Bauer et al., 1998; Gondret et al., 2006), and mice (Desai et al., 2005).

A distinctive characteristic of LBWT neonates is that growth of the brain and heart are protected at the expense of other organs including muscles and liver (Bauer et al., 1998; Desai et al., 2005). Our data are in support of this finding since heart and kidney weights were not affected by feeding status nor energy supplementation. Conversely, although liver mass was less in LBWT pigs, its contribution to total body weight was greater in LBWT compared to NBWT pigs. These data are in agreement with previous observations in pigs suggesting that while liver weight is reduced in LBWT pigs, its contribution to body weight is increased compared to NBWT piglets (De Vos et al., 2016). In addition, feeding the HE diet increased the contribution of the liver to body weight in both LBWT and NBWT pigs. In fact, there is a tight correlation between digestive organ weight and plane of nutrition in many species like rats (Ferrell and Koong, 1986; Burrin et al., 1988), sheep (Burrin et al., 1990; McLeod and Baldwin, 2000) and pigs (Koong et al., 1982; Koong et al., 1983; Liu et al., 2014). This is why it was not surprising that liver mass increased in response to feeding an energy dense diet. The greater contribution of metabolically active digestive organs is a primary cause for increased maintenance energy requirements in animals fed at a high vs those fed at a lower plane of nutrition (Koong et al., 1982; Koong et al., 1983; Burrin et al., 1990; McLeod and Baldwin, 2000), and this may be regarded as competition for energy utilization between digestive organs and muscles (Reeds et al., 1993).

In addition to the greater liver weight that occurred in response to lipid supplementation, fat deposition was greater in the liver of pigs and was not dependent on growth status. Hepatic adiposity occurs in children that undergo a catch-up growth period during childhood (Malpique et al., 2018). In animal models such as rats, feeding a high-fat diet *ad libitum* over 11 weeks increased

liver weight compared to those fed a low-fat diet (Hsu and Yen, 2007; Handjieva Darlenska and Boyadjieva, 2009). Likewise, increase in liver weight as a percentage of bodyweight occurs in LBWT rat pups at 3 weeks of age fed a normal chow diet (Ogata et al., 1985). Similarly, in LBWT pigs fed *ad libitum* between birth and 10 months old, hepatic fat deposition is greater than their NBWT counterparts (Myrie et al., 2011). In these studies however, long term *ad libitum* feeding (10 weeks; (Hsu and Yen, 2007; Myrie et al., 2011) to 10 months (Myrie et al., 2011) compared with two weeks of limited feeding in the current study. Given the short duration of the current study, greater hepatic fat deposition in response to a 25% increase in dietary lipids was unexpected. However, evidence suggest that an increase in hepatic fat deposition is associated with increased risks of steatosis (Permutt et al., 2012), and type 2 diabetes later in life (Cianfarani et al., 2012; Perry et al., 2014). As previously mentioned, pigs were fed for a 14 d, thus we could not ascertain from the current data whether hepatic fat deposition would persist long-term, or whether fat deposition would aggravate thus predisposing neonatal pigs to hepatic diseases.

Early infancy is a critical period for physiological and metabolic adaptations during which a shift from glucose to fatty acids oxidation occurs (Bougneres et al., 1989; Hawdon and Ward Platt, 1993; Sauer et al., 1994). In LBWT infants (Lubchenco and Bard, 1971) and pigs (Chen et al., 2017) a reduction in circulating glucose concentrations occurs immediately after birth, i.e. first 48 h of life. The reduction in glucose concentration could be ascribed to a limited supplies and/or increased glucose utilization (Davis et al., 1997). Our data indicated that during fasting, plasma glucose concentration was reduced in LBWT pigs compared with their NBWT siblings despite a greater glucose rate of appearance. During fasting, only endogenous glucose production contributes to appearance rates. Thus, in spite of a greater glucose production rates in LBWT pigs, glucose concentration remained lower. Following a meal, while glucose rate of appearance was

not different among the groups, LBWT pigs had a modest but persistent reduction in glucose concentration compared to their NBWT siblings. It is important to note that the magnitude of this reduction was less than previously reported in LBWT pigs at birth (Chen et al., 2017). While it could be argued that a postprandial reduction in dietary glucose supplies contributed to the decline in plasma concentrations, this is unlikely in the current study since LBWT and NBWT pigs were fed equal amounts of the same formulas per kg of bodyweight. A likely explanation for this reduction relates to the increased demands for glucose by visceral organ, peripheral tissues and especially the brain which proportionately catabolize more glucose in LBWT neonates due to its larger contribution to body weight (Bier et al., 1977).

Lipid supplementation is clinically prescribed to treat hypoglycemia in LBWT infants (Mestyán et al., 1976; Sann et al., 1981; Sabel et al., 1982; Sann et al., 1982; Bougneres et al., 1989; Sulkers et al., 1993). The effect of lipid supplementation on glucose homeostasis is attributed to enhancing glucose production or sparing glucose from catabolism. Although the effect of fat supplementation in neonatal pigs (Le Dividich et al., 1991) is similar to that in infants of the same age, in the current study, increasing dietary fat supplementation did not affect plasma glucose concentrations. Several factors should be considered to explain the lack of a hyperglycemic response in neonatal pigs in this study. First, in parenterally fed neonates, intravenous lipid emulsions enhance blood glucose concentrations in LBWT infants by improving gluconeogenesis possibly through improved glycerol availability (Mestyán et al., 1976; Sabel et al., 1982). In fact, glycerol accounts for 20% of glucose turnover in newborn LBWT and NBWT infants (Collins et al., 1992; Sunehag et al., 1999), while in very LBWT premature infants this contribution is even higher (Sunehag, 2003). Others, however, concluded that parenteral glycerol infusion does not stimulate gluconeogenesis to the same magnitude as intravenous lipids

suggesting that the stimulatory effect of fatty acids is independent from that of glycerol (van Kempen et al., 2006). In the current study, plasma glycerol concentration and rate of appearance were not different during fasting or after a meal between LBWT and NBWT piglets. In addition, the contribution of glycerol accounted for 15 to 30% of glucose production which is in agreement with previously reported values for NBWT and LBWT infants (Collins et al., 1992).

Although oral lipid administration results in similar improvements in glucose concentrations in LBWT (Sann et al., 1982; Sann et al., 1988; Bougneres et al., 1989) and NBWT (Bougneres et al., 1989) infants, it often involves feeding lipids rich in medium-chain triglycerides. Herein, pigs were fed formulas that contained corn oil and purified animal fat which are composed of long-chain fatty acids. The advantages of feeding medium-chain over long-chain triglycerides are attributed to an improvement (97 vs 83%) in lipid absorption (Roy et al., 1975), and/or reduced (21%) glucose oxidation despite similar glucose appearance rates (Sulkers et al., 1993). Lastly, studies *in vitro* suggest that long chain fatty acids tend to accumulate more than medium chain fatty acids in isolated hepatocytes (Odle et al., 1991). Nevertheless, the response may also be age dependent since oral lipid supplementation in the first day of life (birth to 14h) enhanced blood glucose concentrations in pigs (Le Dividich et al., 1991), whereas in the current study glucose kinetics were determined at 21 d of age. Further studies are needed to elucidate these differences.

In conclusion, we set out to determine whether oral lipid supplementation would enhance growth and improve glucose concentrations in LBWT pigs. Our data suggest that lipid supplementation modestly improved growth of skeletal muscle and the liver, with a concomitant increase in hepatic fat content in LBWT and NBWT pigs. In addition, lipid supplementation had no effect on glucose homeostasis in LBWT and NBWT pigs suggesting that the hyperglycemic response to lipid intake may depend on the route of administration and age of the neonate.

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**Table 2-1.** Ingredients and nutrient composition of the experimental diet

| **Ingredients** | **Low Energy** | **High Energy** |
|---|---|---|
| | *g/100 g, as fed* | |
| Whey protein isolate[1] (90% CP) | 6.12 | 6.11 |
| Lactose | 0.9 | 0.9 |
| FatPack 80[2] | 0.9 | 1.25 |
| Corn oil | 4.34 | 6.01 |
| Water | 86.33 | 84.33 |
| Xanthan gum[3] | 0.1 | 0.1 |
| Vitamin premix[3] | 0.2 | 0.2 |
| Mineral premix[3] | 0.9 | 0.9 |
| Dicalcium phosphate | 0.2 | 0.2 |
| | | |
| Calculated analysis, *units • kg bwt$^{-1}$ • d$^{-1}$* | | |
| ME, kcal | 165 | 205 |
| Crude protein, g | 13.1 | 13.1 |
| Crude fat, g | 12.44 | 17.12 |
| Lactose, g | 4.1 | 4.1 |

[1] Bulk Foods Direct Nutrition (Tempe, AZ).

[2] Milk Specialties (Eden Prairie, MN).

[3] Dyets Inc (Bethlehem, PA). Vitamin premix provided (g/kg): thiamine HCl, 0.1; riboflavin, 0.375; pyridoxine HCl, 0.1; niacin, 1; calcium pantothenate, 1.2; folic acid, 0.13; biotin, 0.02; cobalamin, 1.5; retinyl palmitate, 0.8; cholecalciferol, 0.05; tocopheryl acetate, 8.8; menadione sodium bisulfate, 0.08. Trace mineral premix provided (g/kg): calcium phosphate, dibasic, 187; calcium carbonate, 279; sodium chloride, 85; potassium phosphate monobasic, 155; magnesium sulfate, anhydrous, 44; manganous carbonate, 0.93; ferric citrate, 10; zinc carbonate, 1.84; cupric carbonate, 0.193; potassium iodate, 0.005; sodium selenite, 0.007.

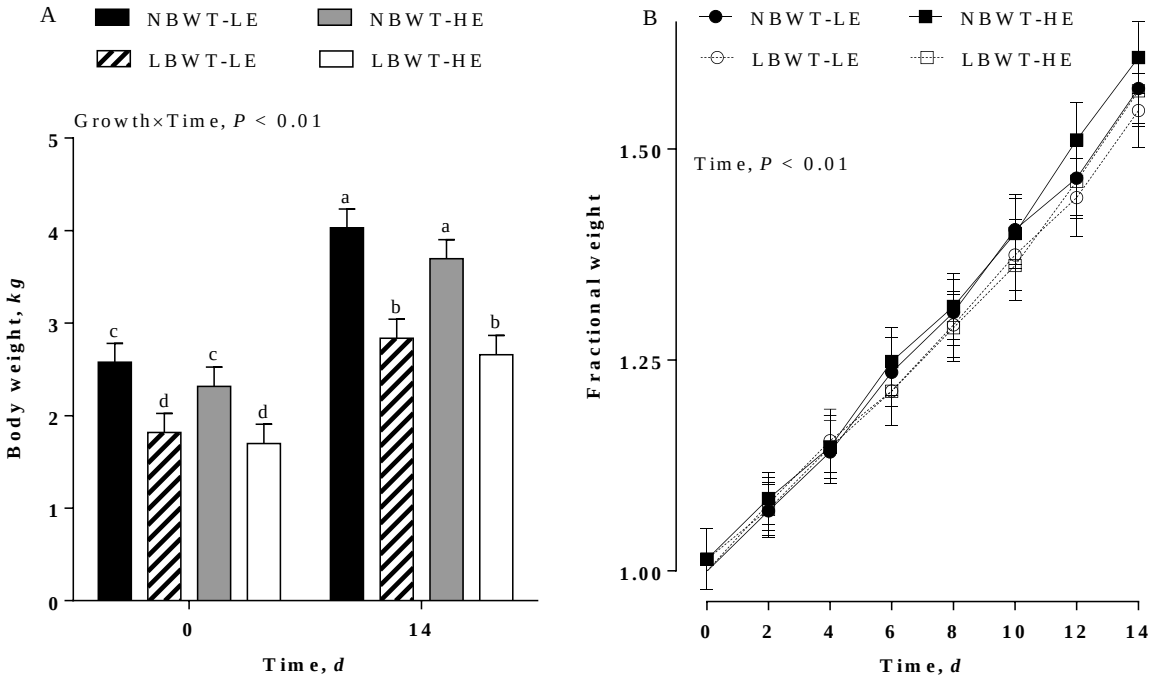


**Figure 2-1.** Bodyweight gain in low (LBWT) and normal (NBWT) birth weight pigs fed a high (HE) and low (LE) energy diet over 14-d. A) body weight and B) fractional weight gain. Results are means ± SE ($n$=6). Values with different letters differ significantly ($P \leq 0.05$)

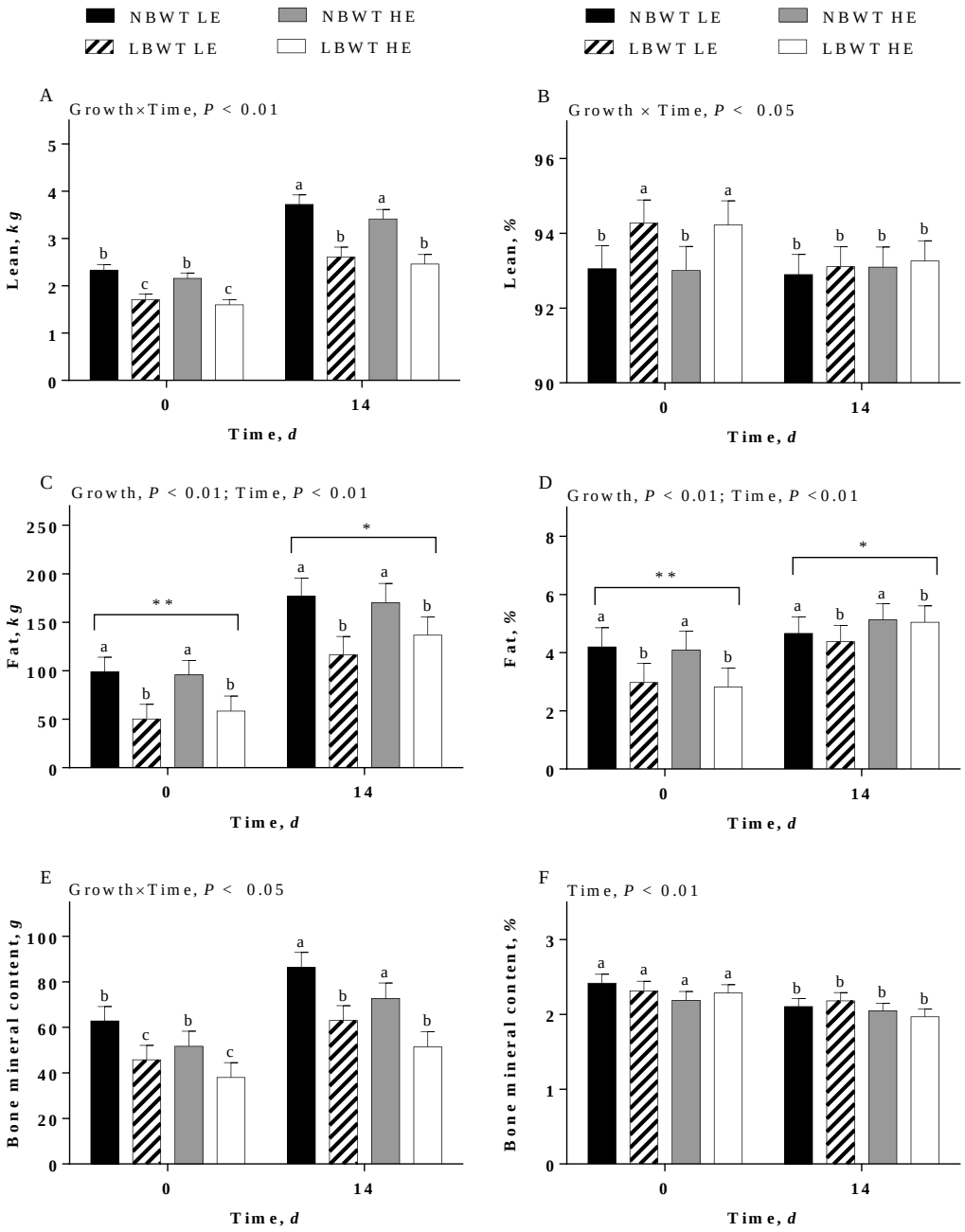


**Figure 2-2.** Growth performance measured by dual energy X-ray absorptiometry in low (LBWT) and normal (NBWT) birth weight pigs fed a high (HE) and low (LE) energy diet. A) absolute lean mass, B) percentage of lean mass to body weight, C) absolute fat mass, D) percentage of fat mass to bodyweight, E) absolute bone mineral content and F) percentage of bone mineral content to bodyweight. Results are means ± SE ($n$=6). Values with different letters differ significantly ($P \leq$ 0.05)

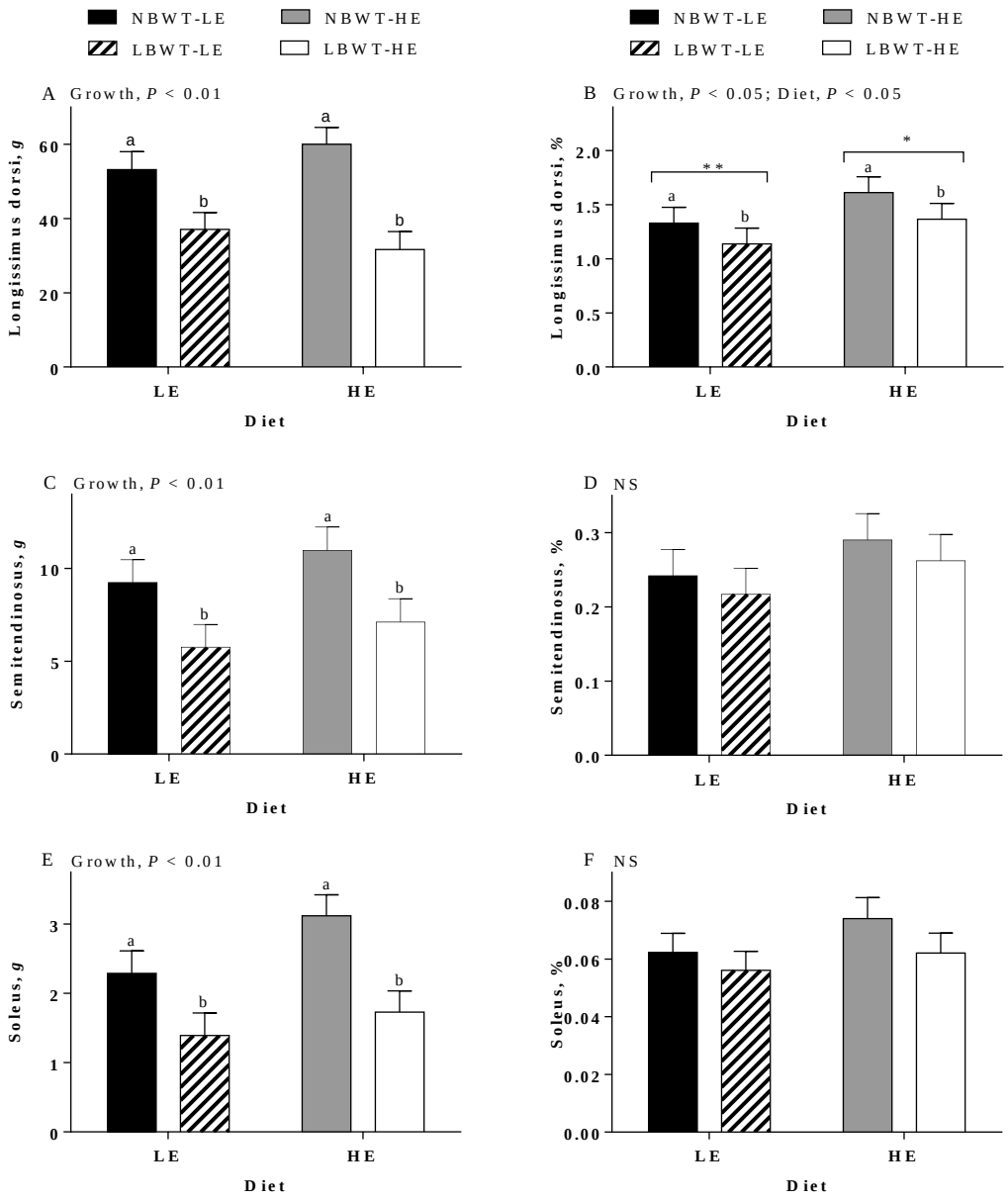


**Figure 2-3.** Muscle weights in low (LBWT) and normal (NBWT) birth weight pigs fed a high (HE) and low (LE) energy diet. A) absolute *longissimus dorsi* (LD) weight, B) percentage of LD weight to body weight, C) absolute semitendinosus (ST) weight, D) percentage of ST weight to body weight, E) absolute soleus weight and F) percentage of soleus weight to body weight. Results are means ± SE ($n$=6). Values with different letters differ significantly ($P \leq 0.05$)

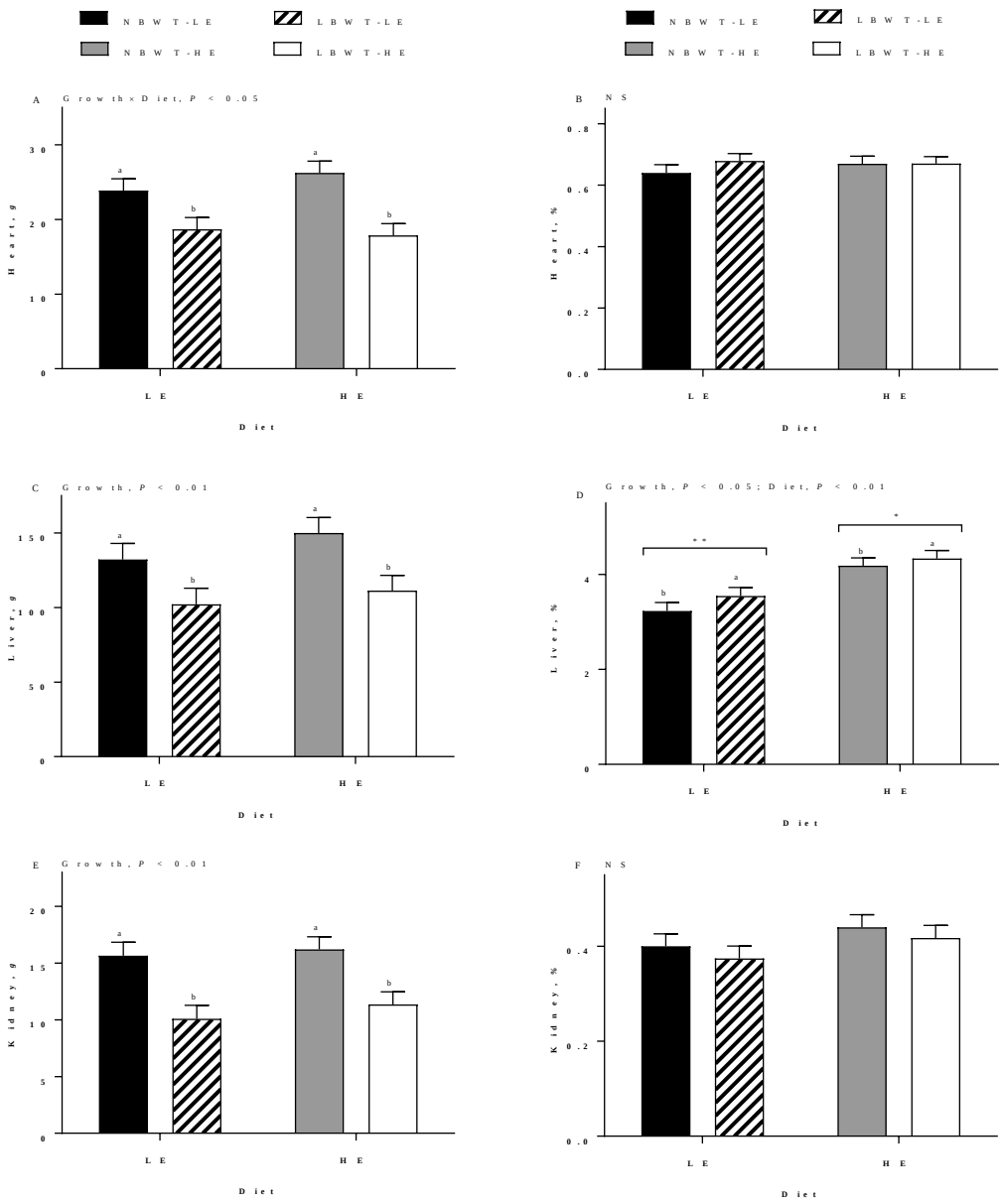


**Figure 2-4.** Organ weights in low (LBWT) and normal (NBWT) birth weight pigs fed a high (HE) and low (LE) energy diet. A) absolute heart weight, B) percentage of heart weight to body weight, C) absolute liver weight, D) percentage of liver weight to bodyweight, E) absolute kidney weight and F) percentage of kidney weight to bodyweight. Results are means ± SE ($n$=6). Values with different letters differ significantly ($P \leq 0.05$)

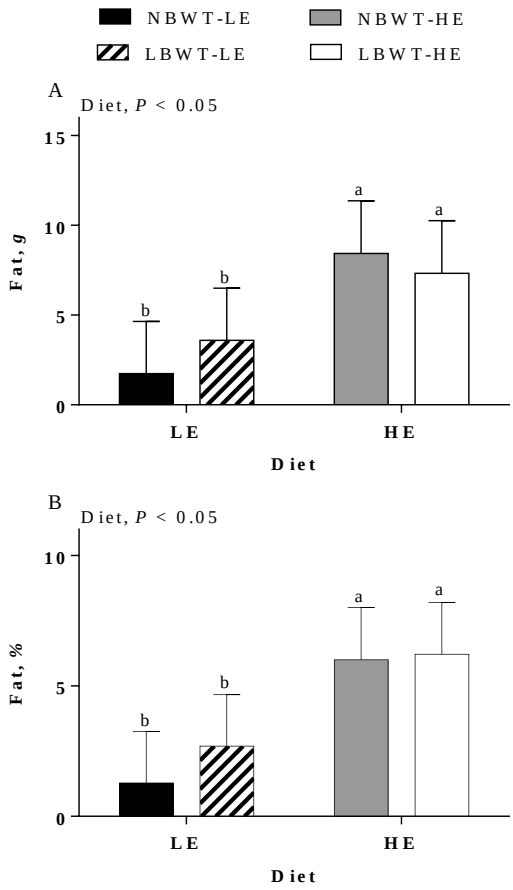


**Figure 2-5.** Fat content in liver of low (LBWT) and normal (NBWT) birth weight pigs fed a high (HE) and low (LE) energy diet. A) absolute liver fat weight and B) percentage of fat to liver weight. Results are means ± SE ($n$=6). Values with different letters differ significantly ($P \leq 0.05$)

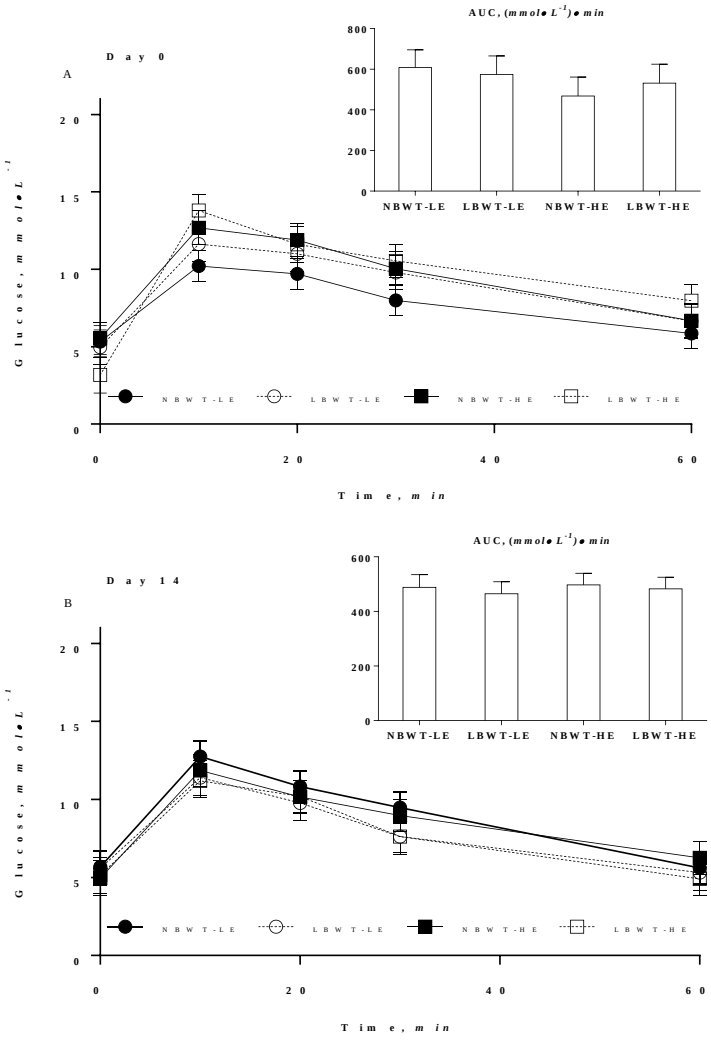


**Figure 2-6.** Intravenous glucose tolerance test (IVGTT) for LBWT and NBWT fed a HE and LE diet. A) IVGTT on day 0 before feeding and B) IVGTT on day 14 after feeding. Results are means ± SE ($n$=6). Values with different letters differ significantly ($P \leq 0.05$).

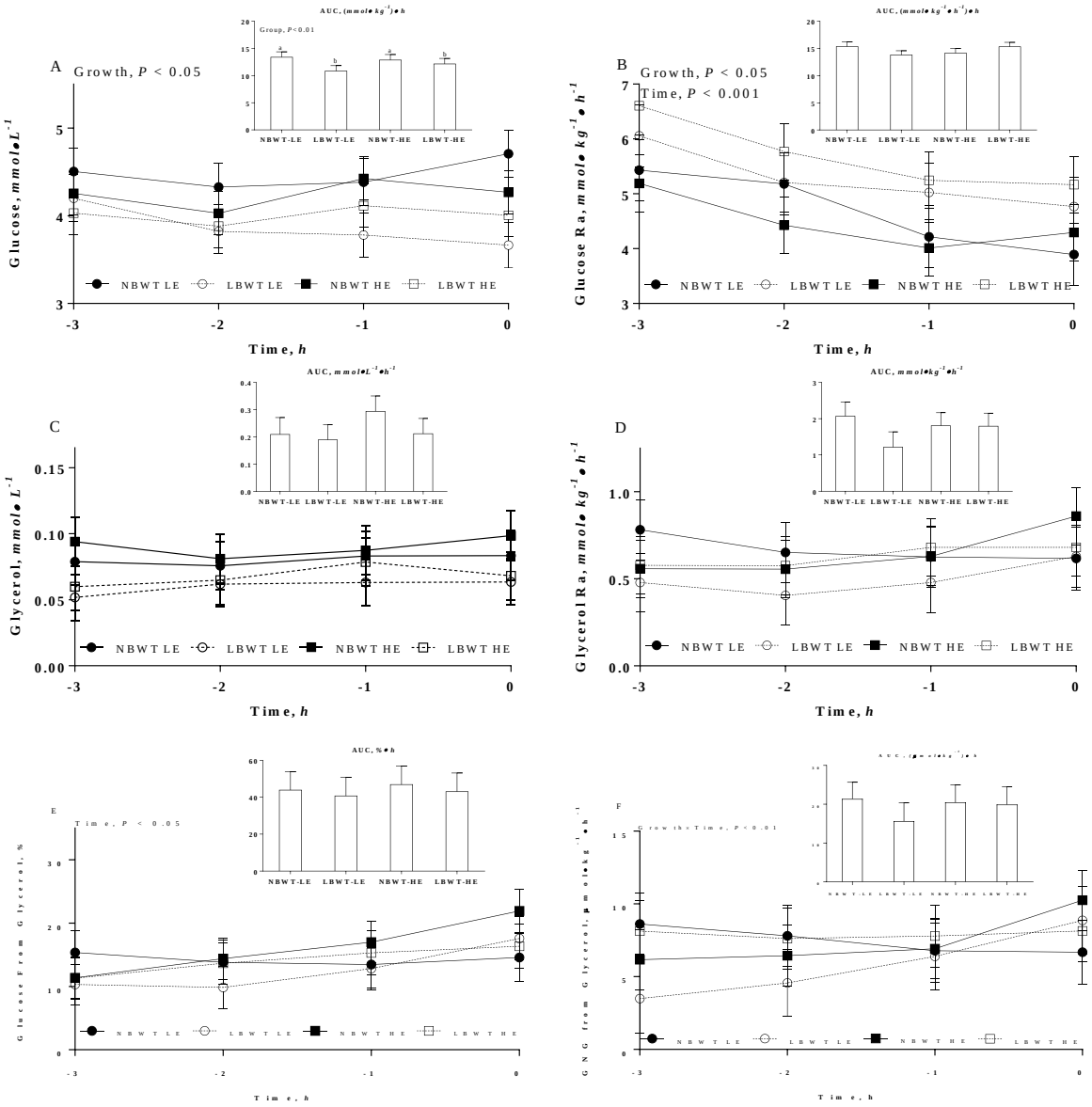


**Figure 2-7.** Fasting plasma glucose and glycerol kinetics over a 4 h period prior to feeding a high (HE) and low (LE) meal in low (LBWT) and normal (NBWT) birth weight pigs. A) glucose concentration, B) glucose rate of appearance, C) glycerol concentration, D) glycerol rate of appearance, E) percentage of glucose produced from glycerol and F) rate of glucose production from glycerol. Results are means ± SE ($n$=6). Values with different letters differ significantly ($P \leq 0.05$).

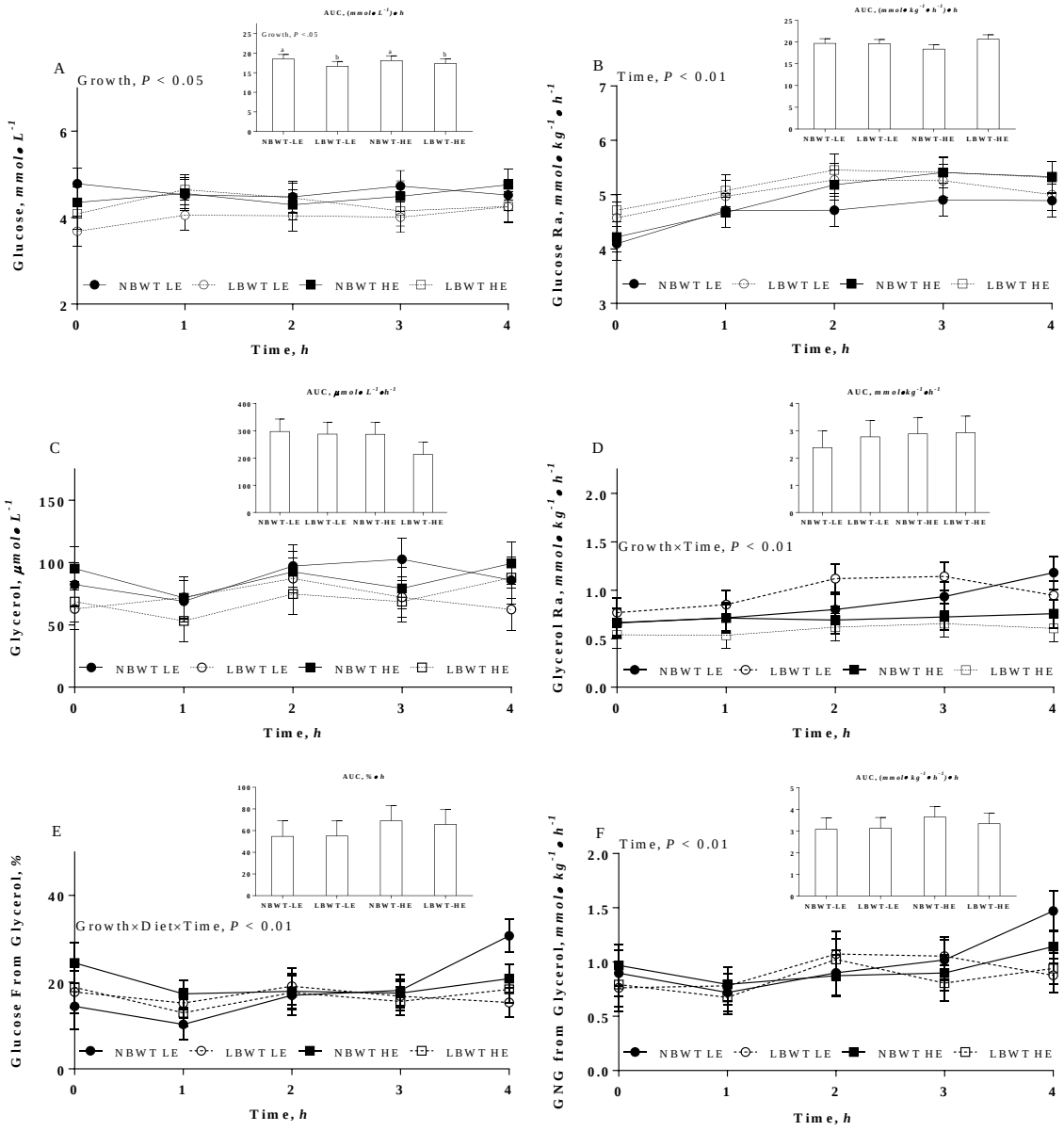


**Figure 2-8.** Plasma glucose and glycerol kinetics over a 4 h period postprandial of a high (HE) and low (LE) meal in low (LBWT) and normal (NBWT) birth weight pigs. A) glucose concentration, B) glucose rate of appearance, C) glycerol concentration, D) glycerol rate of appearance, E) percentage of glucose produced from glycerol and F) rate of glucose production from glycerol. Results are means ± SE ($n$=6). Values with different letters differ significantly ($P \leq 0.05$).

# Chapter 3 Glucose Catabolism in Low Birth Weight Neonatal Skeletal Muscle

**Abstract**

Our previous data show that despite increased glucose rate of appearance, glucose concentration is reduced in low (LBWT) compared to normal (NBWT) birthweight pigs. These data suggest that glucose catabolism may be greater in LBWT pig muscles. The objective of this study was to investigate gene expression of glucose catabolic pathways in skeletal muscle of newborn LBWT and NBWT pigs. Eighteen pairs of male and female newborn LBWT and NBWT pigs were euthanized to collect plasma and *Longissimus Dorsi* (LD) and soleus (SOL) muscles. Plasma and muscle metabolites were measured by GC-MS. Muscle mRNA expression was measured by real-time PCR. Relative mRNA expression of GLUT-4 was reduced in NBWT compared to LBWT greater pigs in both LD and SOL ($P<0.01$). In contrast, mRNA expression of phosphofructokinase (PFKM) and pyruvate kinase (PK2), was greater in NBWT than in LBWT group ($P<0.001$). Pyruvate dehydrogenase (PDK4) mRNA expression was higher in LBWT ($P<0.001$) than NBWT siblings with LD having higher expression of PDK4 ($P<0.05$) than SOL. In addition, mRNA expression of genes related to the serine biosynthetic pathway were reduced in LBWT compared to NBWT pigs in both the LD and SOL ($P<0.07$). mRNA expression of glucose-6-phosphate dehydrogenase (G6PD) was not affected by tissue or growth status. However, phosphoribosyl pyrophosphate synthetase 2 (PRPS2) was reduced in NBWT than LBWT pigs ($P<0.01$). Plasma glucose concentration was greater in NBWT compared to LBWT pigs ($P<0.001$). In spite of the reduction in mRNA expression of serine biosynthetic enzymes, plasma and skeletal muscle concentrations of free serine and glycine did not differ between LBWT and NBWT pigs. Free essential amino acids were reduced in LBWT ($P<0.01$) compared to NBWT in both the SOL and LD, except for isoleucine. In addition, all plasma essential amino acids except for isoleucine were decreased in LBWT compared to NBWT pigs ($P<0.05$). Despite an increase

in mRNA expression of GLUT 4, mRNA expression of genes related to glycolysis and serine biosynthesis were reduced with an increase in PDK4 expression. In conclusion, the increased expression of PDK4 expression concomitant with a reduction in genes related to glycolysis suggest that substrate oxidation may be changed to fatty acid oxidation in the skeletal muscle of LBWT neonatal pigs.

**Keywords:** Low birth weight, Glucose metabolism, glycolysis, skeletal muscle

## Introduction

Low-birth weight is a major concern in animal production (Wu et al., 2006) and perinatal medicine (Resnik, 2002; Ferenc et al., 2014). Infants whose birthweight are below the 10$^{th}$ percentile (Rosenberg, 2008) are considered LBWT and are at higher risks for perinatal morbidity, mortality, developing life-long metabolic diseases, such as obesity, insulin resistance and type II diabetes (Barker, 2000; Godfrey and Barker, 2000; Resnik, 2002). The incidence of LBWT remains high, 10-15% of all births, even in developing countries (Berghella, 2007; Saleem et al., 2011). The domestic pig has the highest prevalence of naturally occuring LBWT that is spontaneous and accounts for up to 20% of the litter (Quiniou et al., 2002; Wu et al., 2006). Due to similarities in growth and metabolism, the domestic pig has been used as a model for infants (Bauer et al., 2003; Caminita et al., 2015).

The occurrence of LBWT not only decreases the chances of survival but also has permanent stunting effects (Widdowson, 1971; Bauer et al., 2003; Wu et al., 2006). Infancy is characterized as the period where the body displays the highest rate of growth compared to any other period in the postnatal life (Reeds et al., 2000). During this period of growth it is dominated by skeletal muscle hypertrophy (Schiaffino et al., 2013). Growth in spontaneously occurring LBWT is asymmetrical, which is a predominate aspect (75%) in LBWT infants (Chiswick, 1985; Bauer et al., 2003; Ferenc et al., 2014). Asymmetrical growth is associated with lower body weight compared to normal birth weight (NBWT) infants (Widdowson, 1971; Williams et al., 1982; Bauer et al., 2003). Another aspect of asymmetrical growth is the disproportionate slow growth of skeletal muscle relative to internal organs, while the brain is fully developed (Bauer et al., 1998; Bauer et al., 2003).

In utero, glucose levels are maintained by trans-placental transfer from the mother. After birth skeletal muscle accounts for ~20% of whole-body glucose metabolism, and up to 40% of the total muscle mass in the body of non-obese individuals (Zurlo et al., 1990; Rolfe and Brown, 1997; Meyer et al., 2002a; Yates et al., 2012). In addition, skeletal muscle and adipose tissue account for ~80% glucose uptake and storage (DeFronzo et al., 1981b; Meyer et al., 2002b). Given that muscle accounts for the majority insulin-stimulated glucose uptake and metabolism (DeFronzo et al., 1981b), it is also considered to be a primary contributor in insulin resistance (Lillioja et al., 1988; Warram et al., 1990). Due to stunted growth LBWT individuals exhibit similar characteristics to obese, insulin resistant individuals in that they have reduced oxidative capacity and increased glycolytic capacity (Jensen et al., 2007; Jensen et al., 2008). In addition, rapid catch up growth and weight gain in early childhood that is associated with LBWT individuals increases the incidence of insulin resistance (McKeigue, 1999; Singhal et al., 2003). Insulin resistant individuals are characterized by a shift in fiber-typing leading to reduced oxidative capacity to increased glycolytic potential (Tanner et al., 2002; Oberbach et al., 2006). However at birth, LBWT piglets exhibit more oxidative compared to glycolytic fibers (Pardo et al., 2013), although at market they exhibit a switch to more glycolytic fibers consistent with insulin resistance (Bee, 2004). In addition, insulin resistance of peripheral tissues is characterized by increased concentrations of insulin in an attempt to clear high amounts of plasma glucose. Children of LBWT have significantly greater fasting glucose and insulin levels during mid to late childhood which resulted in insulin resistance compared to NBWT children (Gupta et al., 2007). Impaired growth of skeletal muscle could be a major contributor to lifelong insulin resistance and glucose intolerance (Brown, 2014), however the changes at birth in skeletal muscle are not well defined.

We have previously demonstrated that despite low glucose concentrations in LBWT pigs, glucose rate of appearance was similar in LBWT compared to NBWT neonates. The hypothesis of this study was that expression of genes related to glucose catabolism in skeletal muscle is greater in LBWT compared to NBWT neonatal pigs. The objective of this study was to profile glucose catabolic pathways in skeletal muscle of LBWT and NBWT pigs.

**Materials and Methods**

*Animals and sample collection.* All procedures were approved by Virginia Tech Institutional Animal Care and Use Committee. Gestating sows had free access to water and fed a corn-soybean based diet daily to meet NRC requirements (Committe of Nutrient Requirements of Swine, 2012). At birth, piglets were weighed and characterized as normal (NBWT) or low (LBWT) birth-weight when body weight was within ±0.5 SD or ≤ 2 SD of the litter mean (D'Inca et al., 2010). Eighteen 1-d old NBWT piglets were matched to eighteen LBWT same-sex siblings. After a 3 h fast, piglets were euthanized for blood and tissue sample collection. Blood samples were centrifuged and plasma extracted. *Longissimus dorsi* (LD) and soleus (SOL) muscles were collected and snap frozen in liquid nitrogen and stored in -80°C freezer for further analysis. Muscle and organ samples were weighed immediately after euthanasia.

*Total RNA extraction.* LD and SOL muscle RNA was extracted and purified by Direct-zol RNA Miniprep Kit (ZYMO Research, Orange, CA). Briefly, 50mg of skeletal muscle was crushed in liquid nitrogen using mortar and pestle and placed in 0.8 ml of Tri-Reagent (ZYMO Research, Orange, CA). Tissues were homogenized using TissueLyser II (Qiagen, N.V.) at 25 *hz* for 2.5 *min*. Once samples were homogenized, 0.2ml of chloroform was added and samples were vortexed and incubated at room temperature for 2 min. After incubation samples were centrifuged for 15 min at 12,000 *g*. After centrifugation the supernatant was added to a new RNase free tube and

equal amounts of ethanol was mixed with the samples (1:1). Mixture was loaded into a Zymo-Spin™ IIC Column and processed according to kit instructions.

*RNA Quantification and cDNA synthesis.* RNA concentrations were determine using the NanoDrop spectrophotometer at 260 nm and 280 nm (Thermo Fisher Scientific, Wilmington DE). Aliquots (20µl) of 200ng•µl$^{-1}$ RNA was made of each sample after concentrations were measured. Using the manufacturer's protocol of High Capacity cDNA Reverse Kit (Applied Biosystems, Foster City, CA), 20µl of the reverse transcriptase mixture was added to sample aliquots to make a 40µl reaction. Samples were placed into the thermocycler (Mastercycler Nexus, Eppendorf, Hauppauge, NY) and ran according to kit protocol.

*Quantitative real-time PCR.* Real-time qPCR was carried out using the ABI 7500 Fast Real-time PCR cycler (Applied Biosystems). First, gene specific master mixes were prepared to produce the following final concentrations in each well: 1X of 2X Fast SYBR Green Master Mix (Thermo Fisher Scientific, Wilmington DE), 10µM of forward and reverse primers and nuclease free water. Final concentration of 10ng•µl$^{-1}$ of cDNA was added in triplicates for each gene on a 96-well plate. 18S was utilized as an internal control gene and also used to normalize quantification of the mRNA target. Relative mRNA expression levels were determined using the $2^{-\Delta\Delta Ct}$ comparative method. Primer sequences are presented in Table 1.

*Glucose and amino acid concentrations.* Plasma glucose and amino acid concentrations were determined as well as tissue amino acids using a GC-MS as previously described. To a known amount of plasma, an equal amount of solution containing [U-$^{13}C_6$]-glucose or [U-$^{13}C$]-amino acids internal standards (Cambridge Isotopes Laboratories, Andover, MA). Tissue samples (100mg) with internal standard (100µl) were homogenized on ice in 0.2M of perchloric acid. Tissue samples were then centrifuged and the supernatant extracted for amino acid quantification

the same as plasma samples using a. Glucose and amino acid concentrations were analyzed by gas chromatography-mass spectrometry (GC-MS). Briefly, the aldonitrile pentaacetate derivative of glucose was formed and ions at *m/z* 314/319 ([U-$^{13}C_6$]-glucose) were monitored in electron ionization mode (Hannestad and Lundblad, 1997). Amino acids were converted to their *t*-butyldimethylsilyl derivatives (Calder et al., 1999). The following ions (*m/z*) were monitored in electron ionization mode: alanine 260, 264; glycine 246, 249; valine 288,294; leucine 302, 309; isoleucine 302, 309; proline 286, 292; glutamine 300, 306; methionine 320, 326; serine 390, 394; threonine 404, 409; phenylalanine 336, 346; aspartate 418, 423; glutamate 432, 438; lysine 329, 336; histidine 440, 449; and tyrosine 466, 468.

*Plasma Insulin.* Plasma insulin concentrations were measured using commercially available porcine insulin ELISA Kit according to manufacturer recommendation (R&D systems).

*Tissue Glycogen.* Tissue glycogen was measured spectrophotometrically (Hammelman et al., 2003). Briefly, 1ml of 1.25M HCl was added to 100mg of tissue and homogenized using TissueLyser II (Qiagen, N.V.). Samples were placed on a heating block for 2h at 90°C and then centrifuged for 5min at 15,000 rpm. Homogenates were neutralized with 1.25 KOH (A. and F., 1985). Concentrations of glycogen equivalents were measured spectrophotometrically, using a modified method for microplate volumes at 340nm in duplicates using 96-well microplates.

*Statistical analysis.* Data were analyzed by PROC MIXED of SAS (Version 9.4, SAS Inst. Inc., Cary, NC). Birth weight was the main effect, and sow and sex were the random effects. When a significant treatment effect was detected, means were compared using Tukey-Kramer post-hoc test. Data were expressed as the least squares means ± SEM and differences considered significance at $P < 0.05$.

**Results**

*Growth.* Birthweight of piglets were lower in LBWT compared to NBWT ($P < 0.01$, Figure 1). *Longissimus dorsi* (LD) and *SOL* weight and their weights as a percentage of bodyweight (Figure 2) were less in LBWT compared to NBWT ($P < 0.001$).

*Plasma Metabolites*. Fasting glucose concentration (Figure 3A) in NBWT pigs was 50% greater than LBWT littermates ($P < 0.001$). Similarly, fasting plasma insulin concentration (Figure 3B) was greater in NBWT compared to LBWT piglets ($P < 0.001$). Plasma essential amino acids concentrations (Table 2), except for isoleucine, were reduced in LBWT piglets ($P < 0.05$). The greatest reduction was for methionine which was 50% less in LBWT than for the NBWT pigs. Concentration of proline, glutamine, glutamate and tyrosine was lower ($P < 0.05$) in LBWT compared to NBWT piglets.

*Muscle Metabolites.* LD glycogen (Figure 4A) was less ($P < 0.05$) in LBWT compared to NBWT piglets, however, glycogen content in the SOL was similar in both groups (Figure 4B). Essential amino acid concentrations in the LD (Table 3) was greater in NBWT compared to LBWT pigs ($P < 0.01$), except for isoleucine which was greater in LBWT than their NBWT siblings ($P < 0.01$).

*Glucose catabolism.* Expression of GLUT4 mRNA was greater in the LD and SOL of LBWT compared to NBWT pigs ($P < 0.01$, Figure 5A). Hexokinase 1 (HK1) expression was similar between the two groups, regardless of tissue (Figure 5B). Hexokinase 2 (HK2) expression (Figure 5C) was greater in the SOL of LBWT compared to their NBWT siblings, however expression was similar in the LD ($P < 0.05$). Phosphofructokinase (PFKM) mRNA expression (Figure 5D) was less in the SOL compared to the LD $P < 0.001$), and was greater for NBWT compared to LBWT in both tissues ($P<0.001$). Expression of pyruvate kinase (PKM2) (Figure

5E) was greater in NBWT compared to LBWT pigs, regardless of tissue ($P < 0.001$). Lactate dehydrogenase (LDH) expression (Figure 5F) was greater in the LD than the SOL, regardless of growth status ($P < 0.001$).

*Pyruvate catabolism*. Pyruvate carboxylase (PC) expression (Figure 5A) was greater in the LD compared to SOL ($P < 0.001$) but was unaffected by growth status. Although pyruvate dehydrogenase (PDHA1) expression (Figure 5B) was not affected by growth status or tissue, mRNA expression of PDK4 (Figure 5C) was greater in LBWT in LD and SOL with the greatest expression in the LD ($P$=0.08, Figure 5H). Phosphoenolpyruvate carboxykinase (PCK2) expression (Figure 5J) was greater in the LD compared to the SOL, however, along with NBWT having higher expression compared to LBWT in both tissues ($P < 0.01$).

*Serine biosynthetic pathway*. Phosphoglycerate dehydrogenase (PHGDH) expression (Figure 6A) was greater for in the NBWT LD muscle compared to SOL, regardless of growth status ($P < 0.08$). Phosphoserine aminotransferase (PSAT1) and phosphoserine phosphatase (PSPH) expression (Figure 6B-C) was less for LBWT compared to NBWT pigs ($P<0.001$) in both muscles.

*Pentose Phosphate Pathway.* Glucose-6-phosphate dehydrogenase (G6PD) mRNA expression was not affected by muscle or growth status (Figure 7A). 6-Phosphogluconolactonase (PGLS) and phosphogluconate dehydrogenase (PGD) expression (Figure 7B-C) was greater in the LD compared to SOL, regardless of growth status ($P < 0.001$). Conversely, phosphoribosyl pyrophosphate synthetase (PRPS2) expression (Figure 7D) was greater for LBWT ($P<0.001$) compared to NBWT group in both muscles.

**Discussion**

Evidence suggests that glucose concentration is reduced in LBWT compared to NBWT neonates in the first few days of life. It is not clear whether this reduction is due to greater degradation in skeletal muscles. In the current study, catabolic pathways that utilize glucose were profiled in the LD and SOL of LBWT and NBWT neonatal pigs.

LBWT neonates, are at a greater risk for disturbed glucose homeostasis due to limited glucogenic substrate availability and increased metabolic demands caused by relatively larger brain size as a proportion of body weight (Platt and Deshpande, 2005; Chacko et al., 2011). Hypoglycemia is common in LBWT newborn infants and piglets (Setia et al., 2006; Chen et al., 2017) and coincides with a reduction in amino acids in LBWT human (Economides et al., 1989) and pig fetuses (Lin et al., 2012). Amino acids play a role in protein synthesis during anabolism but could also be catabolized for energy when other substrates are depleted (Wolfe, 2006). A similar reduction in glucose concentration in spite of similar glucose rate of appearance in slightly older pigs (McCauley et al., 2016) and newborn infants (Kalhan et al., 1986) would suggest that an increase in glucose utilization in peripheral tissues.

Skeletal muscles account for up to 40% of total body mass in adults and about 35% in newborns (Wu et al., 2006) and is a significant site for insulin-stimulated glucose uptake and catabolism (DeFronzo et al., 1981a; Meyer et al., 2002b). Glucose metabolism supports basic physiological functions like glycogen synthesis, or catabolized for energy production and intermediary metabolite synthesis depending upon the metabolic needs of the tissue. In the current study, despite an increase in GLUT4 expression, expression of glycolytic enzymes PFKM and PCM were reduced in LBWT compared to NBWT neonatal pigs, suggesting a reduction in the flux of glucose through glycolysis. PFKM is a key regulator of glycolysis and is the first

committed step of glucose catabolism via glycolysis (Weber, 1977). It is possible that glucose is used for glycogen synthesis (Garcia et al., 2009), however, this is unlikely since in the current study glycogen content was reduced in LBWT pigs compared to their NBWT sibling. However, a greater expression of the oxidative phase of the PPP in LBWT compared to NBWT neonates, could indicate that glucose flux is possibly increased to the PPP. Although it is well known that the oxidative phase of the PPP provides NADPH for other anabolic pathways, and ribose-5-phosphate required for purine and pyrimidine synthesis, it remains unclear what physiological role this may serve in LBWT neonates.

Serine biosynthetic pathway provides serine and glycine. Serine plays a major role as the predominant source of one-carbon units for the synthesis of purines, and in cell proliferation, especially in cancer cells (Warburg, 1956). PHGDH is a regulator of the serine biosynthetic pathway flux and cell proliferation (Locasale et al., 2011; Locasale, 2013; DeNicola et al., 2015). It has also been suggested that through anaplerosis, PCK2 provides carbons from the TCA cycle for serine biosynthesis (Brown et al., 2016). In the current study, expression of PHGDH in the LD, and PSAT1 and PSPH was greater, with a concomitant reduction of PCK2 in the NBWT than LBWT group. While the current data would suggest that the reduction in these enzymes would lead to a reduction in muscle serine concentration, muscle serine concentration was similar in LBWT and NBWT pigs. It is possible that despite the reduction of serine biosynthesis could have occurred, serine could have been synthesized by other tissues, and muscle concentrations would have been maintained through circulation. What our data would suggest is that the reduced expression of these enzymes would have occurred to spare glucose and TCA cycle intermediates. Further studies are needed to elucidate this point.

The entry of glucose carbon to the TCA cycle is controlled by two enzymes, PC and PDH. In this study, PC expression was similar between LBWT and NBWT pigs, indicating that anaplerosis is not affected by birth status. Although PDH expression was also similar between the two groups, expression of PDK4, an inhibitor of PDH, was greater in LBWT compared to NBWT piglets. This increase in PDK4 expression would suggest a greater inhibition of activity PDH (Linn et al., 1969) which would reduce the catabolism of pyruvate in the TCA. This increase further supports the indication that the glycolytic flux of glucose carbons is decreased in the skeletal muscle of LBWT neonates. An increase in PDK4 also indicates a possible shift from glucose to fatty acids oxidation in skeletal muscles (Zhang et al., 2014).

Amino acids play a role in protein synthesis during anabolism but could also be catabolized for energy when other substrates are depleted (Wolfe, 2006). In the current study plasma and muscle free amino acid concentrations were decreased, except for isoleucine, in the LBWT compared to NBWT piglets. The increase in isoleucine concentrations may suggest an enhanced glucose uptake in skeletal muscle of rats (Doi et al., 2005) and in pigs (Zhang et al., 2016), which is consistent with increased GLUT4 expression, but contradicts the reduction in catabolic enzyme mRNA expression. The cause for the greater isoleucine concentration in LBWT pigs remains unknown. However, it is possible that the reduction in amino acid concentrations reflects greater flux of these amino acids into the TCA cycle (Owen et al., 2002). In fact, a shift from a glycolytic to a more oxidative muscle fiber type occurs in LBWT pigs and that this phenotype remains until adult age (Powell and Aberle, 1981), which would explain the greater catabolism of amino acids for energy production. A limitation of the current study was that substrate oxidation was not determined to ascertain whether changes in mRNA expression coincide with changes in substrate utilization by muscles.

In conclusion, although GLUT4 expression was greater in LBWT compared to NBWT neonatal pigs, expression of the glycolytic enzymes that are responsible for the complete oxidation of glucose was reduced. The reduction in mRNA expression serine biosynthesis, and pyruvate entry to the TCA cycle would suggest a sparing mechanism from complete catabolism. Likewise, the greater expression of enzymes in the oxidative phase of the PPP may indicate a similar glucose carbon sparing mechanism. Thus, the reduction in glucose catabolism would suggest a possible shift from glucose oxidation to fatty acid and/or amino acid oxidation.

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**Table 3-1.** Real-Time qPCR primer sequences

| Gene | Direction | Primer Sequence | Accession No. |
|---|---|---|---|
| *18S* | Forward | 5'-GTA ACC CGT TGA ACC CCA T-3' | AY265350 |
| | Reverse | 5'-CCA TCC AAT CGG TAG TAG CG-3' | |
| *GLUT4* | Forward | 5'-AGT GGC TGG GAA GGA AGA AG-3' | NM_001128433 |
| | Reverse | 5'-AAT GAG GAA CCG TCC AAG AA-3' | |
| *HK1* | Forward | 5'-GCA GTG GAA GCC AGC TTT TC-3' | NM_001243184 |
| | Reverse | 5'-GCA TCA TAG TCC CCG CGT TT-3' | |
| *HK2* | Forward | 5'-GTG GAA ATG GAG CGA TGT G-3' | NM_001122987 |
| | Reverse | 5'- GCA ATG CAC TGG ACG ATG TG-3' | |
| *PFK* | Forward | 5'-CTA TGT TGG GGG CTG GAC TG-3' | NM_001044550 |
| | Reverse | 5'-GCA ATG CAC TGG ACG ATG TG-3' | |
| *PKM2* | Forward | 5'-TTC GCA TCT TTC ATC CGT AA-3' | XP_001929104 |
| | Reverse | 5'-CGC CCA ATC ATC ATC TTC T-3' | |
| *PCM* | Forward | 5'-CAG GAG AAC ATC CGC ATC AAC-3' | NM_214349 |
| | Reverse | 5'-ACC AGC AGG GAA TCG TAG TG-3' | |
| *PCK* | Forward | 5'-ATC CGA AAG CTC CCC AAG TAC-3' | NM_00161753 |
| | Reverse | 5'-CAA TCA CCG TCT TGC TTT CTA CTC-3' | |
| *PDK4* | Forward | 5'-CTG GCT CCC TGG TTC CCC GA-3' | NM_001159306 |
| | Reverse | 5'-TTC TGA ACC GAA GTC CAG CAG CT-3' | |
| *PDH* | Forward | 5'-TTA GGT CTT AGC GTC CTC CCC G-3' | XM_003360244 |
| | Reverse | 5'-ACG CAA CAA GCT TCC TGA CCA T-3' | |
| *LDH* | Forward | 5'-TGG AGT GGA GTG AAC ATC GC-3' | NM_001315678 |
| | Reverse | 5'-TCT GGT GGA AAC TGG ATG CAC-3' | |
| *G6PD* | Forward | 5'-GTT CTT TGC CCG CAA CTC CT-3' | XM_003360515 |
| | Reverse | 5'GAG CCC AGT CAT GGC AAG AAT A-3' | |
| *PGLS* | Forward | 5'-CAG CTG AGG ACT ATG CCA AGA A-3' | XM_003123494 |
| | Reverse | 5'-TCC TCC AAA ATG CGC TTC AGA-3' | |
| *PGD* | Forward | 5'-ATT CGA AAG GCC CTC TAC GC-3' | XM_003127557 |
| | Reverse | 5'-AGT CCT CCA CTG CCG ACT TA-3' | |
| *RPE* | Forward | 5'-TAT CTG CAC CTG GAT GTA ATG GAC-3' | XM_003133629 |
| | Reverse | 5'-ATG TGC ATG TCA AAG AAG GGG T-3' | |
| *TKT* | Forward | 5'-CGC CAC CCA GCT ACA AAG TT-3' | NM_001112681 |
| | Reverse | 5'-TGT AGC ACT CGA TGA AGC GG-3' | |
| *PHGDH* | Forward | 5'-CTG GCC GGC GTT GTA AAC-3' | NM_001123162 |
| | Reverse | 5'-GCT TCA GCC AGA CCA ATC CA-3' | |
| *PSAT1* | Forward | 5'-CAA AGT GCA GGC TGG AAA TAA CT-3' | XM_021065150 |
| | Reverse | 5'-CCC CGC CGT TGT TCT TAA-3' | |
| *PSPH* | Forward | 5'-GGG CAT AAG GGA GCT GGT AAG-3' | NM_001243221 |
| | Reverse | 5'-GAC GGG ATG TTG AGC TTT GAA-3' | |

**Table 3-2.** Plasma amino acid concentrations in normal birthweight (NBWT) and low birthweight (LBWT) newborn piglets. Values are means ± Standard Error. *n*=18

| Amino Acid | NBWT | | LBWT | | *P*-Value |
|---|---|---|---|---|---|
| | Mean | SEM | Mean | SEM | |
| Essential, $\mu mol \bullet l^{-1}$ | | | | | |
| Valine | 184[a] | 10 | 106[b] | 10 | <0.001 |
| Threonine | 70[a] | 6.6 | 39[b] | 6.4 | <0.001 |
| Leucine | 108[a] | 10 | 58[b] | 9.9 | <0.001 |
| Isoleucine | 20 | 3.2 | 18 | 3.0 | 0.60 |
| Methionine | 46[a] | 6.8 | 18[b] | 6.5 | <0.001 |
| Phenylalanine | 73[a] | 5.0 | 44[b] | 4.8 | <0.001 |
| Lysine | 167[a] | 22 | 110[b] | 21 | <0.05 |
| Non-Essential, $\mu mol \bullet l^{-1}$ | | | | | |
| Alanine | 277 | 30 | 261 | 29 | 0.47 |
| Glycine | 301 | 34 | 314 | 33 | 0.62 |
| Proline | 467[a] | 49 | 253[b] | 47 | <0.01 |
| Glutamine | 534[a] | 60 | 384[b] | 59 | <0.01 |
| Serine | 105 | 12 | 92 | 11 | 0.24 |
| Aspartate | 22 | 3.4 | 21 | 3.4 | 0.69 |
| Glutamate | 169[b] | 33 | 239[a] | 33 | <0.01 |
| Tyrosine | 176[a] | 23 | 103[b] | 23 | <0.05 |

**Table 3-3.** *Longissimus Dorsi* and soleus amino acid concentrations of normal- (NBWT) and low- (LBWT) birthweight neonatal pigs. Values are means ± Standard Error. $n$=18

| | LD | | Soleus | | | *P*-Value | | |
|---|---|---|---|---|---|---|---|---|
| Amino Acid | NBWT | LBWT | NBWT | LBWT | SEM | Growth | Tissue | Growth×Tissue |
| | | | | *μmol • g of tissue*$^{-1}$ | | | | |
| Essential | | | | | | | | |
| Valine | $140^{b}$ | $96^{c}$ | $183^{a}$ | $111^{c}$ | 9.66 | <0.001 | <0.001 | <0.05 |
| Threonine | $214^{x}$ | $122^{y}$ | $227^{x}$ | $137^{y}$ | 21.8 | <0.001 | 0.44 | 0.98 |
| Leucine | $75^{b,x}$ | $59^{b,y}$ | $91^{a,x}$ | $70^{a,y}$ | 4.98 | <0.001 | <0.01 | 0.55 |
| Isoleucine | $4.4^{b,y}$ | $12^{b,x}$ | $8.6^{a,y}$ | $15^{a,x}$ | 1.53 | <0.001 | <0.01 | 0.66 |
| Methionine | $54^{x}$ | $26^{y}$ | $42^{x}$ | $26^{y}$ | 6.40 | <0.001 | 0.09 | 0.08 |
| Phenylalanine | $65^{b}$ | $49^{c}$ | $79^{a}$ | $51^{c}$ | 3.43 | <0.001 | <0.05 | <0.05 |
| Lysine | $250^{a,x}$ | $164^{a,y}$ | $192^{b,x}$ | $156^{b,y}$ | 17.0 | <0.001 | <0.05 | 0.10 |
| Histidine | $199^{x}$ | $139^{y}$ | $240^{x}$ | $140^{y}$ | 24.7 | <0.01 | 0.39 | 0.41 |
| Non-Essential | | | | | | | | |
| Alanine | $1260^{a}$ | $1319^{a}$ | $1155^{b}$ | $1122^{b}$ | 43.7 | 0.70 | <0.001 | 0.18 |
| Glycine | $1128^{a}$ | $1204^{a}$ | $965^{b}$ | $993^{b}$ | 65.8 | 0.29 | <0.001 | 0.62 |
| Proline | $1461^{x}$ | $797^{y}$ | $1410^{x}$ | $718^{y}$ | 118 | <0.001 | 0.56 | 0.90 |
| Glutamine | $311^{b,x}$ | $260^{b,y}$ | $443^{a,x}$ | $427^{a,y}$ | 24.3 | <0.05 | <0.001 | 0.29 |
| Serine | 482 | 470 | 497 | 453 | 54.6 | 0.27 | 0.96 | 0.54 |
| Aspartate | $1480^{a}$ | $1732^{a}$ | $460^{b}$ | $576^{b}$ | 124 | 0.12 | <0.001 | 0.56 |
| Glutamate | $926^{a}$ | $979^{a}$ | $875^{b}$ | $836^{b}$ | 45.8 | 0.84 | <0.01 | 0.17 |
| Tyrosine | $156^{x}$ | $112^{y}$ | $180^{x}$ | $113^{y}$ | 23.0 | <0.01 | 0.46 | 0.50 |

[a,] Growth, $P$<0.05
[x,y] Tissue, $P$<0.05
[a-c] Growth×Tissue, $P$<0.05

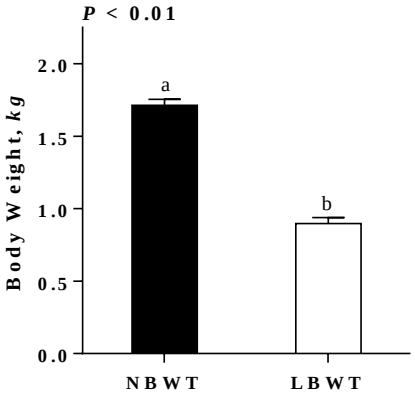


**Figure 3-1.** Body weights of normal- (NBWT) and low- (LBWT) birthweight neonatal pigs. Values are means ± SE. *n*=18. Values with different letters differ significantly ($P \leq 0.05$).

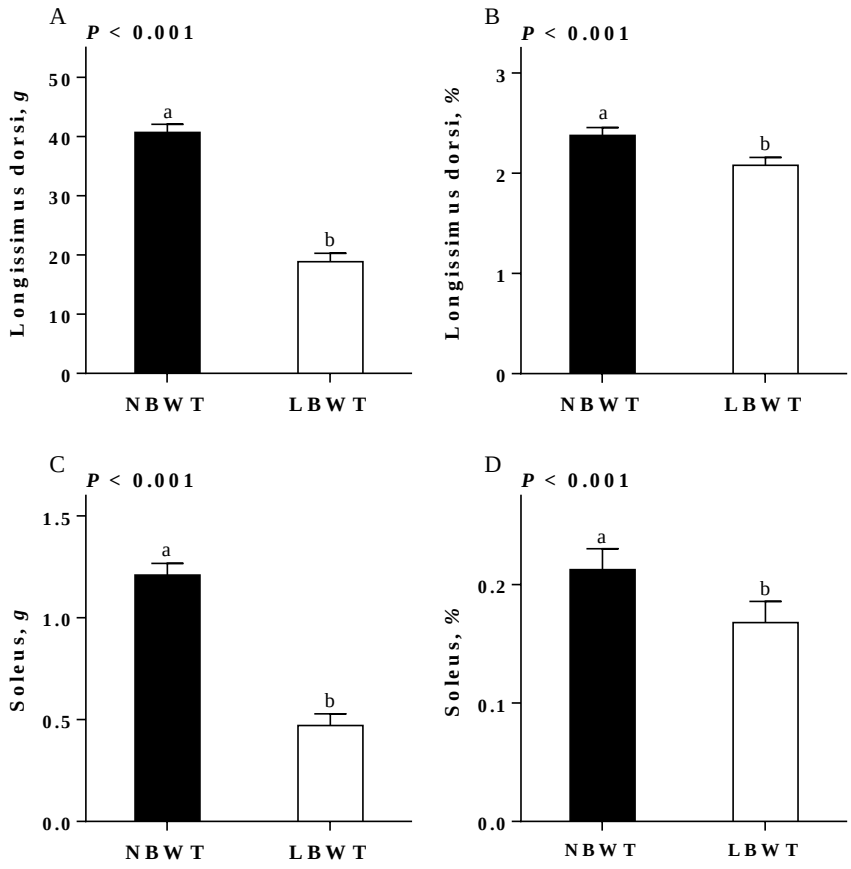


**Figure 3-2.** *Longissimus Dorsi* (LD) and soleus (SOL) muscle weights in normal-(NBWT) and low (LBWT)-birthweight neonatal pigs. A) absolute LD weight, B) percentage of LD weight to body weight, C) absolute soleus weight and D) percentage of soleus weight to body weight. Values are means ± SE. $n$=18. Values with different letters differ significantly ($P \leq 0.05$).

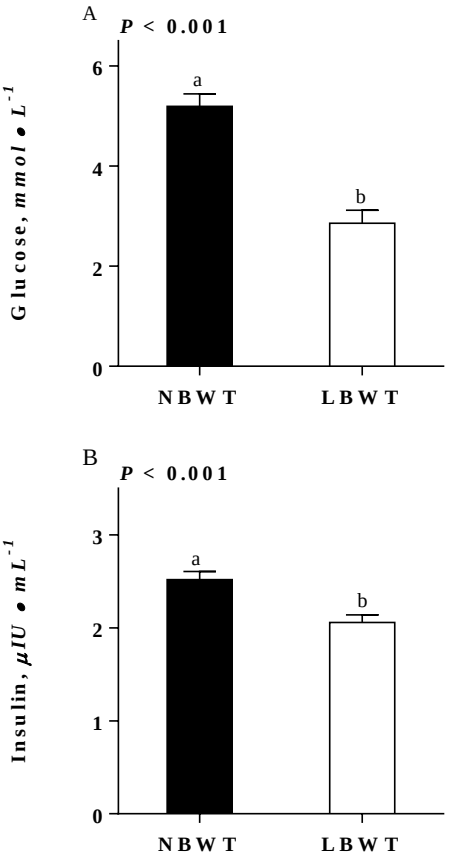


**Figure 3-3.** Fasting plasma glucose A) and insulin B) concentrations in normal (NBWT) and low (LBWT) birthweight neonatal pigs. Values are means ± SE. *n*-18. Values with different letters differ significantly ($P \leq 0.05$).

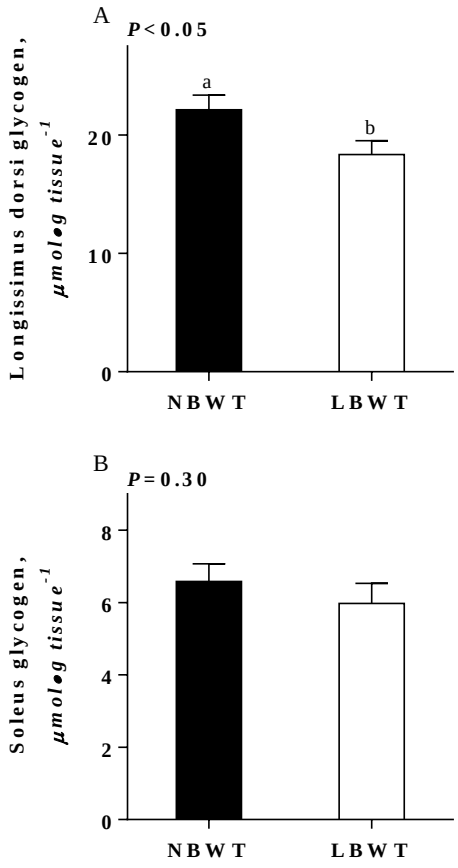


**Figure 3-4.** Total glycogen content in *longissimus dorsi* A) and soleus B) muscles of normal (NBWT) and low (LBWT) birthweight neonatal pigs. Values are means ± SE. *n*=18. Values with different letters differ significantly ($P \leq 0.05$)

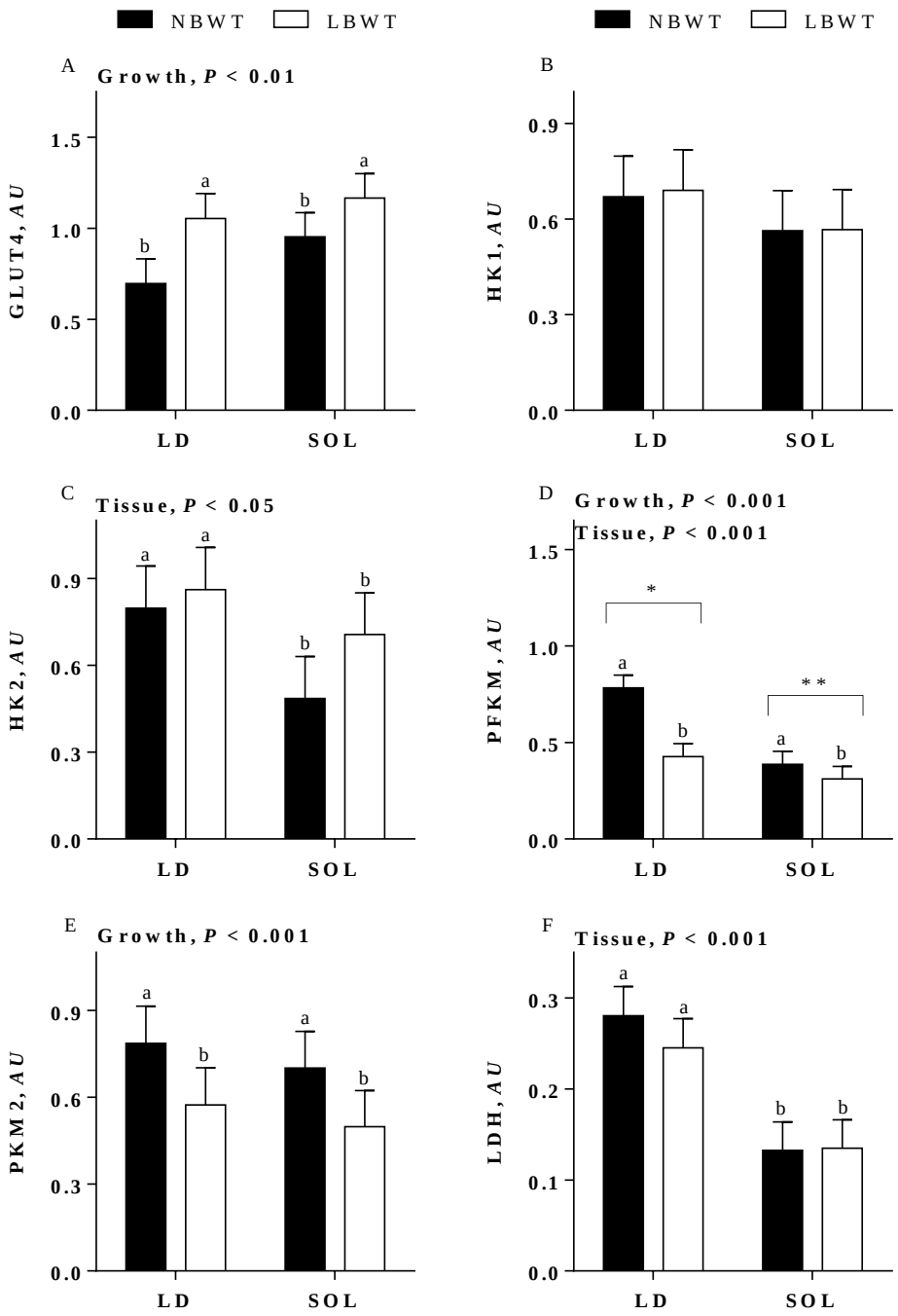


**Figure 3-5.** mRNA expression of genes related to glycolysis in *longissimus dorsi* (LD) and soleus (SOL) of normal (NBWT) and low (LBWT) neonatal pigs. A) sodium glucose transporter 4 (GLUT4), B) hexokinase isoform 1 (HK1), C) hexokinase isoform 2 (HK2), D) phosphofructokinase muscle isoform (PFKM), E) pyruvate kinase muscle isoform 2 (PKM2) and F) lactose dehydrogenase (LDH). Values are means ± SE. *n*=18. Values with different letters differ significantly ($P \leq 0.05$).

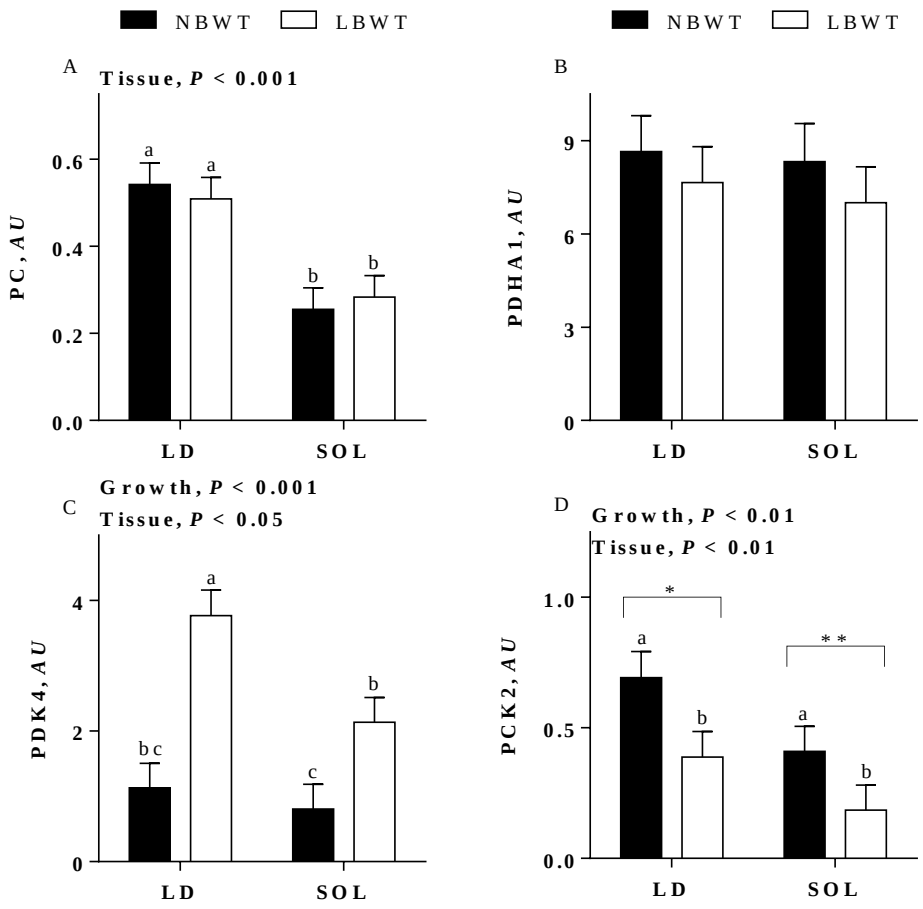


**Figure 3-6.** mRNA expression of genes related to the tricarboxylic acid cycle in *longissimus dorsi* (LD) and soleus (SOL) of normal (NBWT) and low (LBWT) neonatal pigs. A) pyruvate carboxylase (PC), B) pyruvate dehydrogenase arm 1 (PDHA1), C) pyruvate dehydrogenase kinase isoform 4 (PDK4) and D) phosphoenolpyruvate pyruvate carboxykinase isoform 2 (PCK2). Values are means ± SE. *n*=18. Values with different letters differ significantly ($P \leq 0.05$).

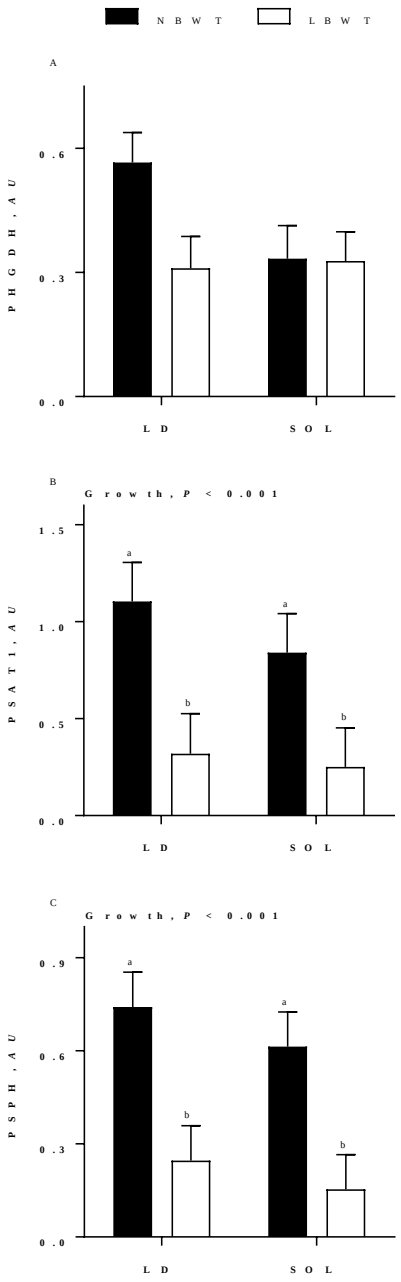


**Figure 3-7.** mRNA expression of genes related to serine biosynthetic pathway in *longissimus dorsi* (LD) and soleus (SOL) of normal (NBWT) and low (LBWT) neonatal pigs. A) phosphoglycerate dehydrogenase (PHGDH), B) phosphoserine aminotransferase 1 (PSAT1) and C) phosphoserine phosphatase (PSPH). Values are means ± SE. *n*=18. Values with different letters differ significantly ($P \leq 0.05$).

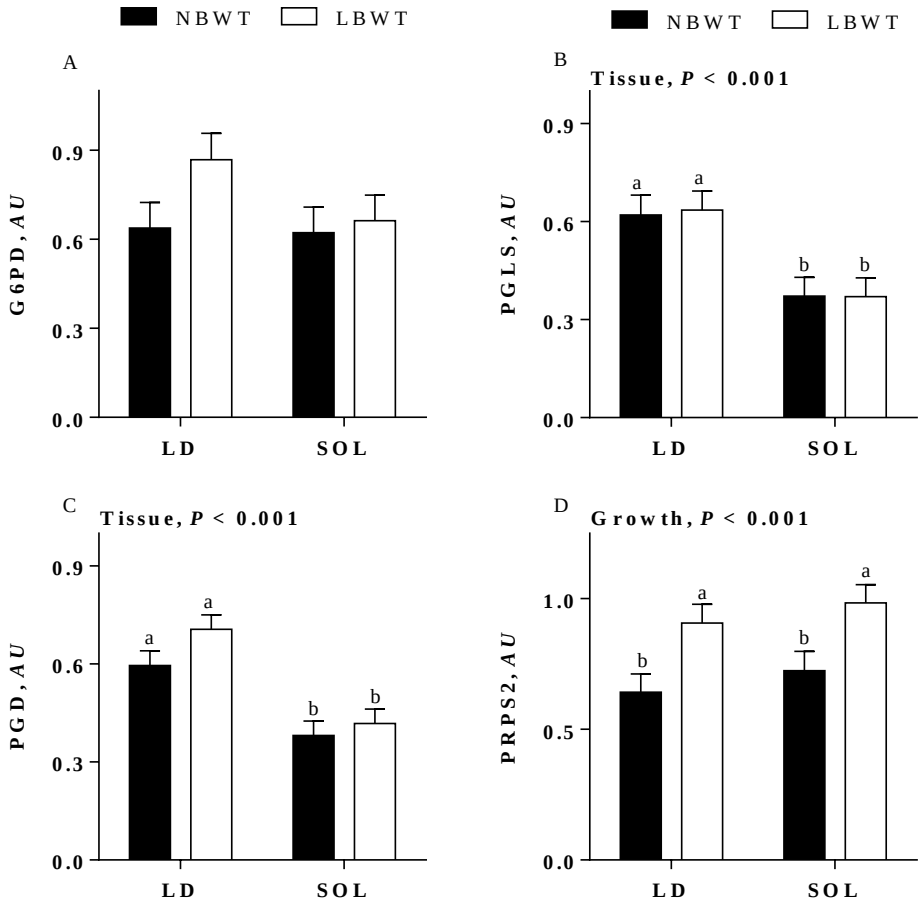


**Figure 3-8.** mRNA expression of genes related to the oxidative phase of the pentose phosphate pathway in *longissimus dorsi* (LD) and soleus (SOL) of normal (NBWT) and low (LBWT) neonatal pigs. A) glucose 6-phosphate dehydrogenase (G6PD), B) phosphogluconolactonase (PGLS), C) phosphogluconate dehydrogenase (PGD) and D) phosphoribosyl pyrophosphate synthetase 2 (PRPS2). Values are means ± SE. *n*=18. Values with different letters differ significantly ($P \leq 0.05$).

# Chapter 4 Metabolic Flexibility in Skeletal Muscle of Low Birth Weight Neonatal Pigs

**Abstract**

Skeletal muscle is a major contributor to whole body glucose homeostasis. We have previously shown that expression of glycolytic enzymes in skeletal muscle is reduced in low (LBWT) birth weight compared to normal (NBWT) birth weight piglets, despite an increase in GLUT-4 expression. These data suggest that glucose catabolism is reduced in skeletal muscle of newborn LBWT. The objective of this study was to assess energy substrate oxidation in skeletal muscle of LBWT and NBWT pigs. Five pairs of male and five pairs of female newborn LBWT and NBWT pigs were euthanized to collect *Longissimus Dorsi* (LD) and soleus (SOL) muscles. Oxidation of [U-$^{14}$C]-glucose was lower ($P$<0.01) in LBWT compared to NBWT pigs both in LD and SOL. The reduction in glucose oxidation coincided with a reduction in phosphofructokinase activity (PFK) which was lower in LBWT compared to NBWT in both SOL and LD ($P$<0.05). Oxidation of [1-$^{14}$C]-pyruvate was less in LBWT compared to NBWT ($P$<0.05) but was not different between LD and SOL muscles. The reduction in pyruvate oxidation occurred with lower citrate synthase (CS) activity in LBWT compared to NBWT ($P$<0.01), regardless of tissue. Oxidation of [1-$^{14}$C]-palmitate was reduced in LBWT compared to NBWT pigs ($P$<0.01), and muscle type had no effect. In addition, acid soluble metabolite production, which represents the incomplete catabolism of palmitate, was not affected by birth weight but was lower in in SOL than in LD ($P$<0.05). The reduction in palmitate oxidation could only be partially attributed to a reduction in β-oxidation since activity of β-hydroxyacyl CoA dehydrogenase (BHAD) was decreased in LBWT compared to NBWT only in the LD ($P$<0.05), whereas activity was similar in the SOL muscle. Oxidative efficiency, which is the ratio of complete to incomplete oxidation of palmitate was lower in LBWT compared to NBWT in both tissues ($P$<0.01). Lastly, pyruvate oxidation in the presence of palmitate was lower in LBWT than NBWT in both the LD and SOL

($P$<0.05). In conclusion, LBWT exhibited a reduction in glucose and fatty acid oxidation, suggesting an early onset of metabolic dysregulation.

**Keywords:** Low birth weight, glucose metabolism, metabolic flexibility, skeletal muscle

## Introduction

Low birth weight (LBWT) neonates are defined as having a birthweight below 2 standard deviations of the mean for gestational age (Chen et al., 2017a) and affects 10-15% of all births in developing countries and 8-9% in the U.S. (Berghella, 2007; Saleem et al., 2011; Martin et al., 2018). The occurrence of LBWT is not only accompanied by permanent stunting effects (Widdowson, 1971; Bauer et al., 2003; Wu et al., 2006) but also associated with a higher risk of developing metabolic diseases later in life such as cardiovascular disease, obesity and type II diabetes (Barker, 2000; Godfrey and Barker, 2000; Resnik, 2002). The neonatal piglet is a good model for studying the human infant due to physiological and metabolic similarities. LBWT in pigs is spontaneous and accounts for up to 20% of the litter (Quiniou et al., 2002; Wu et al., 2006). In addition, the prevalence of LBWT and normal birthweight (NBWT) in the same litter limits parental influence.

During gestation, the fetus relies heavily on carbohydrates as the primary metabolic fuel, accounting for about 80% of energy consumption in utero (DiGiacomo and Hay, 1990; Hay, 1995). After birth, substrate selection for energy is determined by availability during fasting and fed states (Kelley et al., 2002) and the ability to adjust to fuel selection is determined by the balance between oxidative and storage capacity of tissues (Goodpaster and Sparks, 2017). For example, skeletal muscle accounts for ~20% of whole body glucose catabolism making it an essential site for insulin stimulated glucose uptake (DeFronzo et al., 1981) and utilization (Meyer et al., 2002). During insulin resistance or other metabolic disorders such as obesity, skeletal muscles' inability to adapt to changes in response to nutrient availability is known as metabolic inflexibility (Kelley and Simoneau, 1994; Kelley et al., 1999). This inflexibility is also correlated with a shift in muscle fiber types, which results in a shift in oxidative capacity. Adults born of LBWT when challenged

with a hyperinsulinemia-euglycemic clamp experience decreased glucose uptake (Jaquet et al., 2000), suggesting an increased risk for insulin resistance and possible inflexibility.

Due to altered development of skeletal muscle fibers in LBWT, it has consequences on glucose uptake and oxidative capacity. LBWT pigs at birth have more oxidative compared to glycolytic fibers, which is associated with a higher proportion of type I fibers in comparison to type II (Pardo et al., 2013) which may affect glucose uptake, insulin signaling and oxidative capacity. In addition, type I fibers exhibit an increase in the rate of fat oxidation compared to type II muscle fibers (Koves et al., 2008). We previously have shown that reduced expression of genes related to glucose oxidation occurs in LBWT piglets at birth compared to their NBWT siblings. Thus, our hypothesis was that fatty acid oxidation was greater in LBWT compared to NBWT neonatal pigs. The objectives of this study were to: 1) investigate oxidative capacity in skeletal muscle, and 2) determine the metabolic flexibility of skeletal muscle in LBWT piglets.

**Materials and Methods**

*Animals and sample collection.* Gestating sows had free access to water and fed a corn-soybean based diet to meet NRC requirements (Committe of Nutrient Requirements of Swine, 2012). At birth, piglets were weighed and characterized as normal (NBWT) or low (LBWT) birth-weight when body weight was within ±0.5 SD or ≤ 2 SD of the litter mean (Chen et al., 2017a) respectively. Twenty pigs were used from 8 litters. Five male and five female 1-d old NBWT piglets were paired with a LBWT same-sex siblings. After a 3 h fast, piglets were euthanized for blood and tissue collection. *Longissimus dorsi* (LD) and soleus (SOL) muscles were collected for substrate oxidation and enzyme activity. Muscle samples were weighed immediately after euthanasia. All procedures were approved by Virginia Tech Institutional Animal Care and Use Committee.

*Muscle homogenization.* Fresh LD and SO muscle were excised and washed in ice cold PBS. A 100mg of muscle was placed in 200µl of modified SET buffer (25 mM sucrose, 1 mM EDTA, 10 mM tris-HCl and 1mM ATP, pH=7.4) on ice and minced with scissors. More SET buffer was added to produce a final 20-fold dilution (wt:vol). Samples were homogenized in a glass Potter-Elvehjem homogenizer (Omni International, Kennesaw, GA.) with a motor-driven pestle. Sample homogenates were used for substrate oxidation and enzyme activity.

*Skeletal muscle enzyme activity.* Determination of muscle phosphofructokinase (PFK), citrate synthase (CS) and β-hydroxyacyl-CoA dehydrogenase (BHAD) activity was done spectrophotometrically. Briefly, PFK activity was measured by adding 30µl of sample homogenate was mixed with assay buffer consisting of 12mM $MgCl_2$, 400mM KCl, 2mM AMP, 1mM ATP, 0.17 mM NADH, 0.0025 mg•ml$^{-1}$ Antimycin, 0.05 mg•ml$^{-1}$ Aldolase and 0.05 mg•ml$^{-1}$ GAPDH in 100mM of Tris Buffer (pH=8.2). After a background reading, 3mM fructose-6-phosphate was added to initiate the reaction. Activity of PFK was measured at 340nm for 7 minutes at 37°C and expressed as µmol•min$^{-1}$•mg of tissue$^{-1}$. CS activity was measured by adding 10µl of muscle homogenate to a 170µl solution containing Tris buffer (0.1M, pH 8.3), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) (1mM, in 0.1M in Tris buffer), and oxaloacetate (0.01M, in 0.1M Tris buffer). After a background reading, 30µl of 3mM acetyl CoA was added to initiate the reaction to form citrate and Coenzyme A (CoA). Absorbance was measured at 405nm at 37°C every 12 sec for 7 min on a spectrophotometer (SPECTRAmax PLUS 384, Molecular Devices Corporation, Sunnyvale California). Activity of CS was determined from the reduction of DNTB by CoA and reported as µmol•min$^{-1}$•mg of tissue$^{-1}$. Activity of BHAD was determined from the oxidation of NADH to NAD. A 35µl of tissue homogenate was added to 190µl of buffer containing 0.1M triethanolamine, 5mM EDTA tetrasodium salt dihydrate, and 0.45mM of NADH. After a

background reading, 15µl of acetoacetyl CoA was added to initiate reaction. Absorbance was measure at 340nm every 12 seconds for 6 minutes at 37°C and maximum BHAD activity was reported as µmol•min$^{-1}$•mg of tissue$^{-1}$.

*Substrate oxidation in skeletal muscle.* Substrate oxidation in LD and SOL were analyzed as previously described using radio-labeled tracers (Hickson-Bick et al., 2000; McMillan et al., 2015). Briefly, fatty acid oxidation was assessed using [1-$^{14}$C]- palmitate (American Radiolabeled Chemicals, St. Louis, MO.) to quantify $^{14}CO_2$ production and $^{14}$C-labeled acid-soluble metabolites (ASM). Tissue samples were incubated in 0.5 µCi•mL$^{-1}$ of [1-$^{14}$C]- palmitate for 1 h then acidified with 200µl perchloric acid (45%) for 1 h to liberate $^{14}CO_2$. Labeled $CO_2$ was trapped in a tube containing NaOH and then placed in a scintillation vial with 5 ml scintillation fluid and counted (LS 4500, Beckman Coulter). Acidified media were collected to determined ASM enrichment with $^{14}$C. Glucose and pyruvate oxidation were measure with similar methods to that of fatty acid oxidation with the exception of a substitution of [U-14C]-glucose and [1-$^{14}$C]-pyruvate for [1-$^{14}$C]-palmitate. Metabolic flexibility was assessed from pyruvate oxidation in the presence or absence of palmitate.Metabolic flexibility was measured as the ratio of $CO_2$ production from labeled pyruvate to $CO_2$ production with labeled pyruvate in the presence of unlabeled palmitate. Oxidative efficiency, which represents complete and incomplete products of fatty acid oxidation, was calculated from the ratio of $CO_2$ divided by ASM.

**Results**

*Growth.* Birthweight (Figure 1) of piglets was as expected, less for LBWT compared to NBWT pigs ($P < 0.001$). Absolute weights of the LD and SOL (Figure 2A and C) were lower in LBWT compared to NBWT piglets ($P < 0.001$). LD weight as a percentage of bodyweight (Figure

2B) was lower in LBWT compared to NBWT group ($P < 0.01$), however SOL as a percentage of bodyweight (Figure 2D) was similar between the two groups.

*Substrate oxidation.* The rate at which $^{14}CO_2$ was produced from the oxidation of [U-$^{14}$C]-glucose (Figure 3A) was reduced in muscle of LBWT compare to NBWT pigs for both muscles ($P < 0.01$). Production of $^{14}CO_2$ from [1-$^{14}$C]-pyruvate (Figure 3B) was decreased in LD and SOL of LBWT compared to NBWT siblings ($P < 0.05$). Complete fatty acid oxidation, represented by $^{14}CO_2$ production from [1-$^{14}$C]-palmitate (Figure 3C), was reduced in LD and SOL of LBWT compared to NBWT group ($P < 0.01$). Acid soluble metabolites (Figure 3D) were reduced in the SOL compared to the LD, regardless of growth status ($P < 0.05$). Oxidative efficiency (Figure 3E) was greater for NBWT compared to LBWT pigs for both muscles ($P < 0.01$). Metabolic flexibility (Figure 3F) was lower in muscles of LBWT compared to NBWT pigs indicating a less flexible tissue ($P < 0.05$).

*Enzyme activity.* Activity of PFK (Figure 4A) was reduced in LBWT compared to NBWT pigs in both LD and SOL ($P < 0.05$). Similarly, activity of CS (Figure 4B) was reduced in LBWT compared to NBWT neonates in LD and SOL muscles ($P < 0.01$). However, activity of BHAD (Figure 4C) was reduced in LBWT compared to NBWT piglets in the LD with no difference between LBWT and NBWT siblings in the SOL ($P < 0.05$).

**Discussion**

Several studies demonstrate that alterations in intrauterine nutrient supply have large impacts on glucose homeostasis after birth, ultimately leading to type 2 diabetes and obesity in adulthood (Valdez et al., 1994; Rich-Edwards et al., 1999). We have previously shown that the expression of glycolytic enzymes was reduced in skeletal muscles of LBWT compared to NBWT pigs. In the current study radio isotopic tracers were used to ascertain whether the changes we

previously observed in the expression of glycolytic enzymes would translate to changes in substrate oxidation.

Under normal conditions, skeletal muscle metabolic plasticity allows oxidation of different substrates based on availability. For example, during insulin-stimulated conditions glucose uptake, oxidation, and storage are increased while lipid oxidation is suppressed. Under fasting conditions, lipid uptake and oxidation is increased while glucose oxidation is suppressed (Dimitriadis et al., 2011). However, it is not yet clear how nutrient deficiencies in utero would alter metabolism in the first hours after birth. In the current study, LBWT neonatal pigs had decreased rates of glucose oxidation compared to NBWT siblings, which agrees with our previous data in which a reduction in glycolytic enzymes was reported. Regulation of glycolysis and glucose oxidation are controlled mainly through the rate limiting enzymes PFK and PDK4 (Zhang et al., 2014; Ausina et al., 2018). When PFK is inactive the concentration of glucose 6-phosphate increases which causes a feedback inhibition on hexokinase, decreasing the rate at which glucose is oxidized through glycolysis (Lowry et al., 1964; Wilson, 2003). The decrease in in PFK activity in the skeletal muscle of LBWT suggests a reduction in glucose oxidation (Ausina et al., 2018), which coincides with the reduction in glycolytic flux seen in the current study. In addition to the reduction in glucose oxidation, pyruvate oxidation was lower in LBWT which correlates to a decrease in PDH activity (Abdel-aleem et al., 1995). A decrease in PDH activity is associated with an increase in PDK4 expression (Zhang et al., 2014). PDK4 binds to the E1 subunit of PDH inhibiting its phosphorylating activity on pyruvate (Gudi et al., 1995). Our previous data suggest LBWT had a 4-fold increase in PDK4 expression compared to NBWT pigs in skeletal muscle. This increase in PDK4 is seen in LBWT rats at 6 months of age (Selak et al., 2003). The expression of PDK4 is altered during states of fasted and fed and correlate to the activity of the enzyme as

well (Harris et al., 2001). This would suggest that the increase PDK4 expression correlates with an increase in activity, causing the decrease in PDH activity and concomitant decrease in pyruvate oxidation. This data is consistent with normal fasting conditions. Not only does increased PDK4 activity result in decreased glucose oxidation during fasting, but it is also a major regulator of a switch from glucose oxidation to fatty acid oxidation (Dimitriadis et al., 2011; Muoio, 2014).

Under fasting conditions fatty acid uptake and oxidation is enhanced with a reduction in glucose oxidation (Gray and Kim, 2011; Longo and Mattson, 2014). However, in the current study the inability of fatty acids to increase and spare glucose is inconsistent with normal fasting conditions in oxidation was reduced in LBWT pigs. In individuals that exhibit insulin resistance, fatty acid oxidation is decreased during fasting (Kelley and Mandarino, 1990; Kelley et al., 1999). In the current study the rate of fatty acid oxidation was determined by the use of long chain fatty acid (LCFA) palmitate. The rate of LCFA oxidation is dependent upon the enzyme carnitine palmitoyltransferase (CPT-1) in order to shuttle LCFA across the mitochondria membrane as acylcarnitine (Dagher et al., 2001; Bruce et al., 2007). Decreases in CPT-1 activity results in lipid accumulation and insulin resistance (Dobbins et al., 2001). In LBWT rats, key genes that regulate fatty acid metabolism have lower expression than NBWT rats (Germani et al., 2008). At 6 months of age, the mRNA expression of CPT-1 is decreased in LBWT compared to NBWT pigs (Li et al., 2015). In addition, the decrease in LCFA oxidation capacity is seen during insulin resistance (Colberg et al., 1995; Simoneau et al., 1999). Associated with a decrease in fatty acid oxidation is the increase in lipid accumulation in muscle (Kase et al., 2005). This indicates that the decrease in fatty acid oxidation could lead to an increase in lipid accumulation in LBWT neonates later in life predisposing them to metabolic diseases. The decrease in fatty acid oxidation, in part could be due to the use of the LCFA instead of a medium chain fatty acid (MCFA). During the oxidation

of MCFA they can pass freely through the mitochondrial matrix and do not require CPT1 (Dagher et al., 2001; Bruce et al., 2007). In insulin resistant individuals, increased utilization of fatty acids was seen with MCFA instead of LCFA (Harmancey et al., 2013). The maturation and development of LBWT infants is slower than NBWT infants (Ismail and Chang, 2012) and may have implications on the ability to oxidize LCFA in skeletal muscle. In addition to decreased fatty acid oxidation, BHAD activity was lower in LBWT LD muscle, further elucidating the decreased fatty acid oxidation. However, ASM between groups were not different, although ASM were higher in the LD compared to the SOL. This is also seen in adults with diabetes (Koves et al., 2008). CS activity plays a role in substrate oxidation regulation. During fasting when fatty acid oxidation is increased CS inhibits glucose oxidation by increase citrate concentration which has an inhibitory effect on PFK (Spriet, 2014). This inhibitory effect on PFK decreases glucose oxidation. In the current study, CS activity was decreased in LBWT pigs in both the LD and SOL. In 3-month old LBWT rats, a reduction in TCA cycle intermediates resulted in decreased glucose and fatty acid oxidation (Selak et al., 2003). A reduction in TCA cycle intermediates also resulted in the decrease in fatty acid oxidation in insulin resistant individuals (Koves et al., 2008). CS activity is also a key marker for mitochondrial number and content (Blomstrand et al., 1997; Larsen et al., 2012). A reduction in CS activity in LBWT indicate a reduction in the mitochondrial number. The reduced capacity of glucose and fatty acid oxidation can be explained by the reduction in mitochondrial density in LBWT neonatal pig muscle and not likely due to the activity of BHAD. The failure to increase fatty acid oxidation during fasting is indicative of metabolic inflexibility (Kelley and Mandarino, 2000) and could result from differences in fiber type (Essen et al., 1975).

LD in NBWT pigs is a mixed fiber with mainly type IIb fibers (Karlsson et al., 1993). In LBWT pigs however, the expression of type IIb fibers in the LD was lower compared to NBWT

siblings (Chen et al., 2017b), however the percentage of type II fibers are still more than type I (Pardo et al., 2013). Insulin-stimulated glucose metabolism is affected by fiber type and associated with a decreased proportion of type I fibers and increased type II fibers in obesity and type II diabetes (Lillioja et al., 1987; Takahashi et al., 1993; Nyholm et al., 1997; Simoneau and Kelley, 1997; Levin et al., 2007; Stuart et al., 2013). These changes in fiber type are also associated with decreased oxidative capacity in obesity and type II diabetes (Simoneau and Kelley, 1997). In the current study, our data are consistent with the findings from these studies and even at birth LBWT piglets have decreased oxidative metabolism in both the SOL and the LD. These changes could be indicative of a larger proportion of type II fibers to type I in LBWT piglets at birth.

In obese adults a decreased response to fatty acid oxidation during fasting and increased insulin resistant after a meal result in metabolic inflexibility (Kim et al., 2000). In the current study the metabolic flexibility was decreased in LBWT compared to NBWT siblings, indicating more inflexibility. This inflexibility coincides with the inability to increase fatty acid oxidation during fasting in the LBWT skeletal muscle. The failure to increase the oxidation of fatty acids during fasting conditions is likely a key mechanism that leads to fat accumulation in LBWT individuals and predisposing them to metabolic disturbances later in life.

In the current study we examined metabolic flexibility of skeletal muscle during fasting in LBWT neonatal pigs at birth. Our data indicated that glucose and fatty acid oxidation was reduced in the LD and SOL muscles of LBWT pigs resulting in metabolic inflexible. This indicates an inability to effectively switch between metabolic substrates during fasting and possibly leading to the accumulation of fat in skeletal muscle. The inability to effectively increase fatty acid oxidation during fasting conditions in the skeletal muscle of LBWT neonatal pigs at birth may predispose them to metabolic disturbances later in life.

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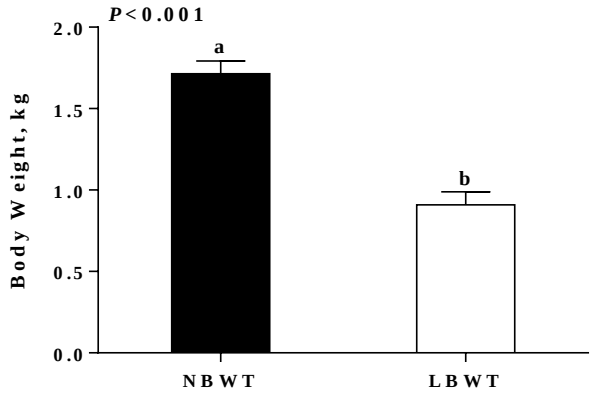


**Figure 4-1.** Body weights of LBWT and NBWT newborn pigs. Values are means ± SE ($n$=10). Values with different letters differ significantly ($P \leq 0.05$).

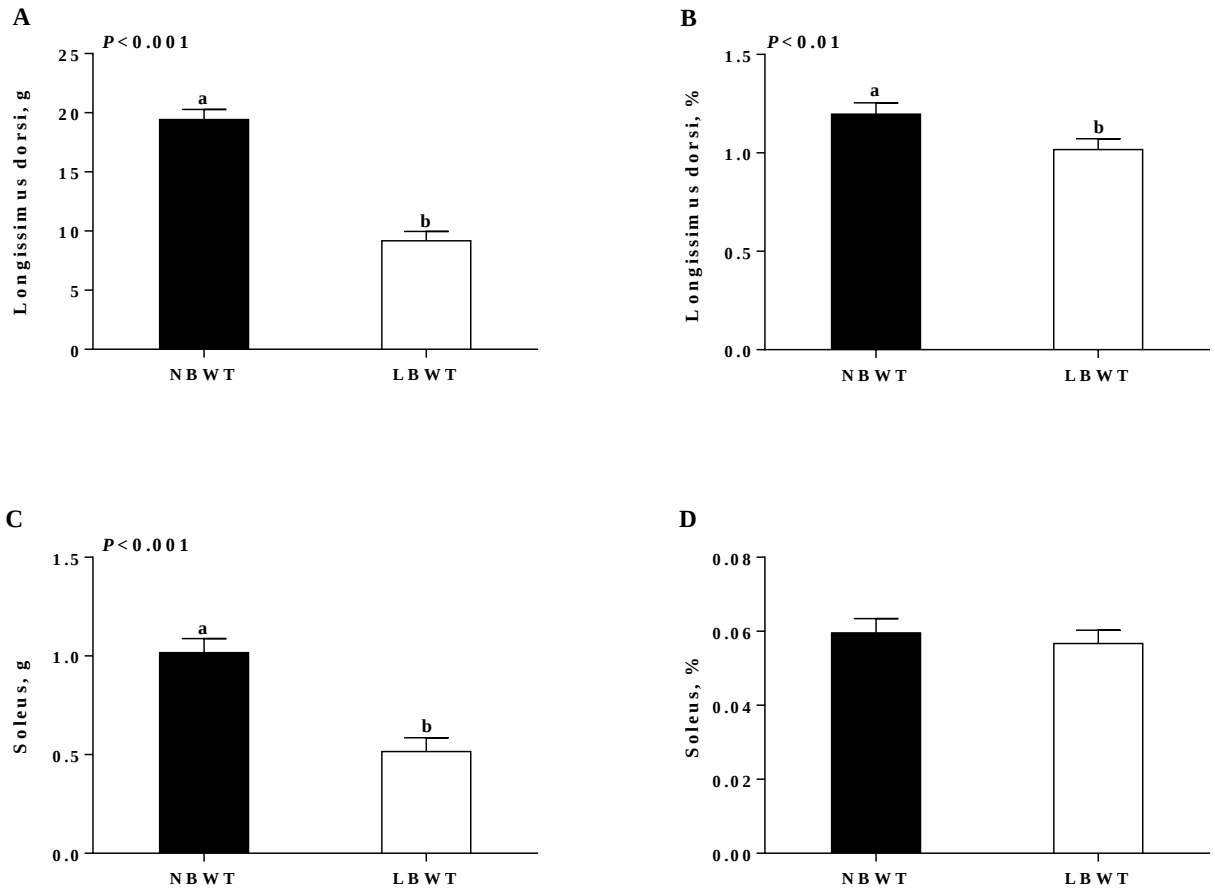


**Figure 4-2.** *Longissimus dorsi* (LD) and soleus muscle weights in LBWT and NBWT newborn pigs. A) absolute LD weight, B) percentage of LD weight to body weight, C) absolute soleus weight and D) percentage of soleus weight to body weight. Values are means ± SE ($n$=10). Values with different letters differ significantly ($P \leq 0.05$).

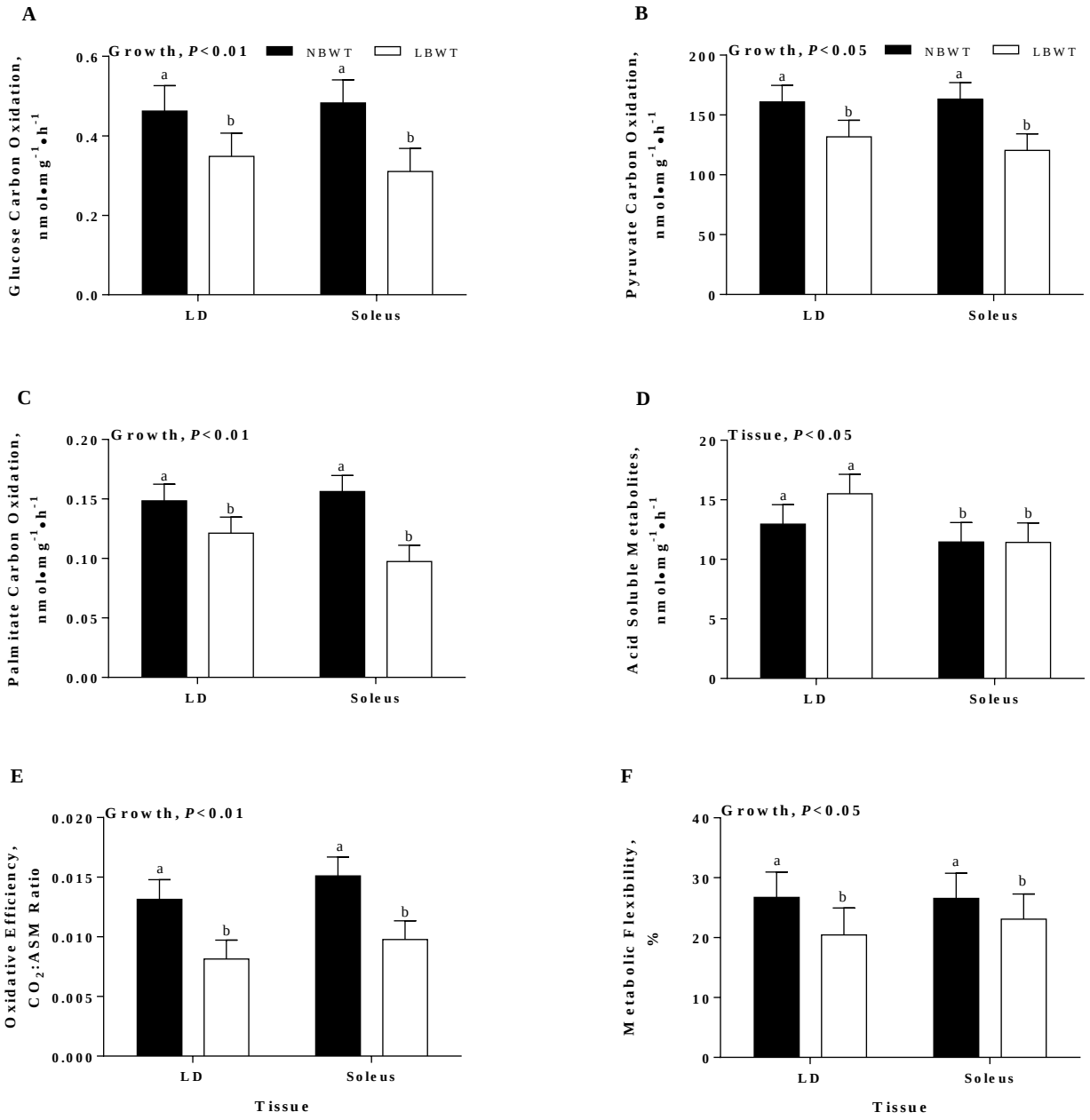


**Figure 4-3.** Substrate oxidation in *longissimus dorsi* (LD) and soleus muscle of LBWT and NBWT newborn pigs. A) $CO_2$ production from oxidation of [U-$^{14}$C]-glucose, B) $CO_2$ production from oxidation of [1-$^{14}$C]-pyruvate, C) $CO_2$ production from oxidation of [1-$^{14}$C]-palmitate, D) acid soluble metabolites measured in acidified media from oxidation of [1-$^{14}$C]-palmitate, E) ratio of labeled $CO_2$ production from oxidation of [1-$^{14}$C]-pyruvate to labeled $CO_2$ in the presence of unlabeled palmitate. Values are means ± SE ($n$=10). Values with different letters differ significantly ($P \leq 0.05$).

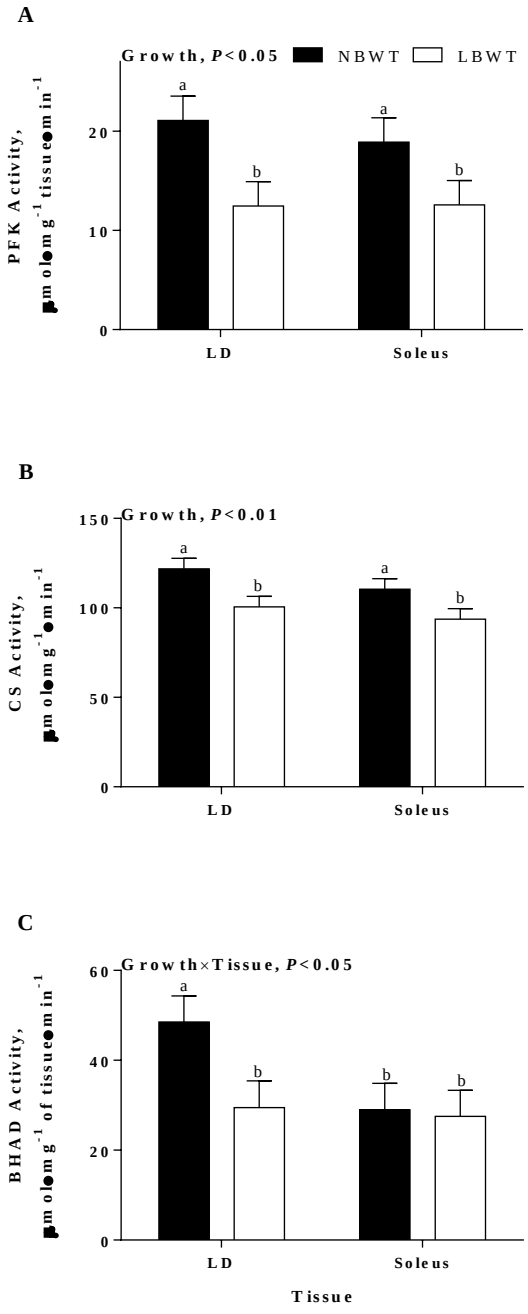


**Figure 4-4.** Enzyme activity in *longissimus dorsi* (LD) and soleus muscle of LBWT and NBWT newborn pigs. A) phosphofructokinase (PFK), B) citrate synthase (CS) and C) β-hydroxyacyl CoA dehydrogenase (BHAD). Values are means ± SE ($n$=10). Values with different letters differ significantly ($P \leq 0.05$).

## Chapter 5 Summary

Low birth weight (LBWT) neonates experience impaired muscle growth and development, leading to not only impaired postnatal growth but increased risk for developing metabolic diseases later in life such as obesity and type 2 diabetes. Since skeletal muscle accounts for a majority of insulin-stimulated glucose uptake and utilization, it becomes one of the primary defects in insulin resistance and metabolic diseases. At birth LBWT is characterized by decreased glucose concentrations along with decreased body fat content at birth. Gaps in our knowledge exist in relation to the metabolic changes that occur in LBWT neonates. Understanding such changes may be beneficial to maximizing growth and improving health outcomes. The aim of this dissertation was to investigate changes in growth performance and glucose production through increasing dietary energy, as well as investigate the changes in glucose catabolism and metabolic flexibility in skeletal muscle in LBWT neonates by using the piglet as the animal model.

The overall hypothesis of this dissertation was that low glucose production and high catabolism of glucose in skeletal muscle predisposes LBWT neonatal pigs to slower postnatal growth. We sought to determine the changes in growth performance and body composition along with the changes in glucose and glycerol kinetics when dietary energy was increased. We also determined changes in gene expression related to glucose catabolism and changes in substrate oxidation in the skeletal muscle of LBWT and NBWT neonatal pigs.

Increasing dietary energy in LBWT and NBWT neonatal pigs by increasing lipids did not improve overall growth. However, an increase in the percentage of LD to body weight was seen in LBWT and NBWT pigs fed a HE diet. In addition, feeding a HE diet

increased liver fat deposition in both LBWT and NBWT pigs. Glucose rate of appearance after a meal was not different in LBWT compared to NBWT siblings, regardless of diet. However, glucose concentration was lower in LBWT compared to NBWT neonatal pigs fed either HE or LE diet. In addition, glucose concentration in LBWT was lower than NBWT during fasting, regardless of diet fed. However, glucose rate of appearance in LBWT neonates was higher during fasting than that found in NBWT siblings, regardless of diet. This would suggest that during fasting glucose uptake is increased in peripheral tissues.

In the second study, expression of genes related to glucose catabolism were profiled in the LD and soleus muscle of NBWT and LBWT neonatal pigs. The expression of genes related to glycolysis were lower in LBWT than NBWT in both the soleus and LD. In addition, genes related to the serine biosynthetic pathway was lower in LBWT compared to NBWT in both the LD and soleus. In contrast, genes related to the pentose phosphate pathway were slightly increased in LBWT compared to NBWT. PDK4 expression, a major regulator of substrate oxidation in skeletal muscle was increased in LBWT compared to NBWT. Overall this data suggested a switch from glucose oxidation in skeletal muscle to more fatty acid oxidation.

The third aimed to investigate the changes in substrate oxidation in both the LD and soleus of LBWT and NBWT neonatal pigs. Glucose oxidation was lower in LBWT compared to NBWT in both the soleus and LD along with a decrease in PFK activity, coinciding with the gene expression found in the second study. However, fatty acid oxidation was also decreased in LBWT neonatal pigs in both the soleus and LD compared to NBWT siblings. Along with decreased fatty acid oxidation, BHAD activity was

decreased in LBWT compared to NBWT in the LD but no change in the soleus. However, acid soluble metabolite which represents incomplete oxidation of fatty acids were not different between LBWT and NBWT. Citrate synthase activity was lower, suggesting that the decrease in fatty acid oxidation was not due to the decrease in BHAD but to the decrease in citrate synthase activity.

Further studies are required to investigate the changes in glucose production during fasting and feeding and determine where increased glucose uptake is occurring in peripheral tissues. In addition, current studies focused on the utilization of glucose and the oxidation of long chain fatty acids in skeletal muscle, it is necessary to assess medium chain fatty acids as well as the contribution of amino acids. It is also necessary to assess underlying mechanism that regulate the changes from glucose oxidation to fatty acid oxidation. Finally, more research is needed to seek nutritional manipulation that will stimulate growth and enhance glucose production in order to increase energy supply to the peripheral tissues without hypoglycemia in LBWT neonatal pigs.