Branched-Chain Amino Acid Metabolism in the Neonatal Pig

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Abstract

Branched-chain amino acids (BCAA) are a group of essential amino acids consisting of leucine, isoleucine, and valine. Leucine, in particular, has signaling functions affecting protein and energy metabolism. Plasma leucine concentration is positively correlated with obesity and associated metabolic disorders. We set out to test the hypothesis that metabolic dysfunction from high fat diets precedes dysfunctional BCAA metabolism. First, BCAA were supplemented to neonatal pigs for 4 weeks to evaluate whether the anabolic signaling function of leucine could increase muscle growth when fed for a longer duration than in previous studies. Neither normal pigs nor low birth weight pigs, which have naturally impaired muscle growth, grew better in response to BCAA supplementation, despite low birth weight pigs expressing less of the leucine sensing protein Sestrin2 in skeletal muscle. Furthermore, high plasma BCAA concentrations caused by the experimental diets had no effect on adiposity, liver fat accumulation, or expression of genes related to fatty acid synthesis, mitochondrial biogenesis, or energy expenditure in the pigs' livers. Having produced strong evidence that long term BCAA supplementation neither improves lean growth nor causes abnormal fat metabolism, we then tested whether fat supplementation changes BCAA metabolism. Pigs were fed milk replacer formula with either low energy (Control), or high energy from long-chain fatty acids (LCFA) or medium-chain fatty acids (MCFA) for 22 days. Although high fat diets did not increase plasma BCAA concentrations, the MCFA diet in

particular caused metabolic changes which could lead to fatty liver disease and decreased oxidative BCAA disposal. Expression of fatty acid synthesizing genes were increased in the livers of pigs fed MCFA formula compared to Control and LCFA formula. Oxidation of α-ketoisocaproic acid was decreased in liver homogenate of pigs fed MCFA and LCFA formulas compared to Control. Additionally, hepatic oxidation of α-ketoisovalerate was decreased, and plasma concentration of α-ketoisovalerate was consequently increased, in pigs fed MCFA formula compared to Control, with LCFA formula causing intermediate results. In future research, it would be valuable to feed high MCFA formula for a longer period of time to determine whether nonalcoholic fatty liver disease will develop, and whether plasma BCAA concentrations will increase due to decreased oxidation. Overall, these studies concluded that long term BCAA supplementation does not increase muscle growth in neonatal pigs, but there is also no indication that they cause obesity or dysfunctional fat metabolism. On the other hand, high fat diets cause impairments in BCAA catabolism which may precede elevated plasma BCAA concentrations.

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General Audience Abstract

Branched-chain amino acids (BCAA) are essential amino acids which are abundant in plant and animal proteins. In addition, the BCAA leucine has functions in protein and energy metabolism. Leucine consumption induces a signal to build new muscle protein. However, leucine concentration is also higher in blood plasma of obese individuals than in non-obese individuals, which has caused uncertainty regarding the safety of leucine consumption. In order to demonstrate that leucine does not cause obesity, we set out to test the hypothesis that high fat diets cause decreased breakdown of BCAA. In the first study, we tested whether one month of BCAA supplementation could increase muscle growth in neonatal pigs. Neither normal pigs nor low birth weight pigs, which have naturally impaired muscle growth, grew better in response to BCAA supplementation, despite low birth weight pigs expressing less of a leucine sensing protein in skeletal muscle. Furthermore, BCAA supplementation caused higher BCAA concentrations in blood plasma, but did not cause pigs to gain more fat, or cause any changes in liver fat metabolism. Having produced strong evidence that BCAA supplementation neither improves lean growth nor causes abnormal fat metabolism, we then tested whether fat supplementation changes BCAA metabolism. Pigs were fed milk replacer formula which was either low calorie (Control), or high calorie from animal fat, which is rich in long-chain fatty acids (LCFA) or high calorie from coconut oil, which is rich in medium-chain fatty acids (MCFA). Although high fat diets did not increase blood

plasma BCAA concentrations, the MCFA formula in particular caused changes which could lead to fatty liver disease and decreased breakdown of BCAA. Genes which synthesize new fatty acids were increased in the livers of pigs fed MCFA formula compared to those fed LCFA and Control formulas. Furthermore, liver samples taken from pigs fed the MCFA and LCFA formulas were less able to fully break down metabolites of leucine compared to pigs fed the Control formula. In addition, liver samples from MCFA fed pigs were less able to fully break down metabolites of the BCAA valine, which led to higher concentrations of that metabolite in the blood plasma of pigs fed MCFA formula compared to pigs fed LCFA or Control formula. In the future, it would be valuable to feed a high MCFA formula for a longer period of time to determine whether nonalcoholic fatty liver disease will develop, and whether blood plasma BCAA concentrations will increase due to decreased breakdown. Overall, these studies concluded that long term BCAA supplementation does not increase muscle growth in neonatal pigs, but there is also no indication that they cause obesity or dysfunctional fat metabolism. On the other hand, high fat diets cause impairments in BCAA breakdown which may lead to elevated BCAA concentrations in blood plasma.

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Introduction

Infants who are unable to consume breast milk are often given milk replacement formula. However, ample evidence suggests that formula fed infants most often fall short of meeting normal growth rates as compared to their breastfed counterparts. In addition, it is well established that reduced growth in the first few weeks or months in life could have long-term consequences especially as it relates to metabolic health. One area that deserves further examination is whether growth of infants consuming primarily formulas could be enhanced using functional nutrient supplementation. Among those, branched-chain amino acids (BCAA) have received most attention for their ability to enhance muscle protein accretion.

Further research is required to establish whether the effect of BCAA supplementation on muscle growth in neonates is sustained long-term. Although the BCAA leucine has been shown to increase protein synthesis acutely following supplementation, it is important to know whether leucine promotes lean growth when fed long-term in milk-replacer formulas. Of special interest was whether BCAA supplementation would enhance the growth of low birth-weight (LBWT) infants. Infants afflicted with this condition have impaired muscle growth, caused in part by decreased rates of protein synthesis. The innate growth deficits of LBWT pigs make them the optimal model to study potential treatments to improve muscle growth. Improved understanding of the benefits of BCAA supplementation will lead to milk-replacer formulas which maximize early lean growth. The overarching goal of this research is to provide evidence of the benefits and safety of BCAA supplementation in infant formula.

Gaps in knowledge also exist regarding the relationship between BCAA and metabolic disease. The association has been subject to research and speculations since plasma BCAA

concentrations were reported to be elevated in obese adults (Felig, 1970). Oxidative disposal of BCAA rely on common pathways with fat oxidation, leading to the theory that BCAA competes with fat oxidation and results in adiposity and liver fat accumulation. Furthermore, fat accumulation may cause metabolic dysfunction which leads to decreased BCAA oxidation. This research aimed to determine whether high blood BCAA concentration precedes metabolic dysfunction or whether metabolic dysfunction causes a reduction in BCAA disposal, causing greater BCAA concentrations.

Controlled trials in animals are a means of explaining the positive correlation between plasma BCAA and obesity. In one of the most influential studies supporting BCAA as the cause of metabolic disease, BCAA supplementation decreased glucose tolerance in rats consuming a high fat diet compared to those consuming high fat diet alone (Newgard et al., 2009). However, BCAA did not affect adiposity, and insulin resistance was dependent on high fat diet. In other experiments, BCAA are beneficial for prevention of adiposity (Freudenberg et al., 2012; H. Li et al., 2012a; Vianna et al., 2012) and improved glucose homeostasis (Binder et al., 2013; Eller et al., 2013; Zhang et al., 2007). Furthermore, preexisting metabolic diseases often result in greater circulating BCAA concentrations. For example, mice fed a high fat, high fructose diet developed nonalcoholic fatty liver disease which decreased the liver's ability to oxidize BCAA, while BCAA supplementation did not increase hepatic fat accumulation (Sunny et al., 2015). BCAA concentrations are elevated in plasma of genetically obese and insulin resistant rodents (Maida et al., 2017; She et al., 2007; Zhou et al., 2019), and high fat, high sugar diets increased BCAA concentration in porcine plasma (Polakof et al., 2018) and livers (Frano et al., 2019). Whether elevated plasma BCAA concentrations precede obesity and irregular fat metabolism remains unknown.

The overall hypothesis of this research was that metabolic dysfunction from high fat diets precedes dysfunctional BCAA catabolism. The objectives of this research were to: 1) Determine whether BCAA supplementation to LBWT pigs increases protein synthesis and affects hepatic metabolism, and 2) Determine whether high fat diets cause hepatic dysfunction or disturbances in BCAA catabolism.

Chapter 1. Literature Review

Branched-Chain Amino Acids

Overview

The branched-chain amino acids (BCAA) are a group of three essential amino acids with hydrophobic, methylated side-chains. The three BCAA are leucine, isoleucine, and valine (Figure 1.1). Isoleucine only differs from leucine in that it is methylated at the β-carbon rather than the γ-carbon, and valine contains one carbon fewer than leucine. The Structural similarities of the BCAA impart them with similar functional properties within proteins and allow them to share many of the same transport proteins and catabolic enzymes. Animals are not capable of synthesizing BCAA, although they are able to re-aminate their corresponding keto acids back to the parent amino acid. Therefore, BCAA account for three of the ten essential amino acids which animals must consume an adequate amount of for normal protein synthesis to occur.

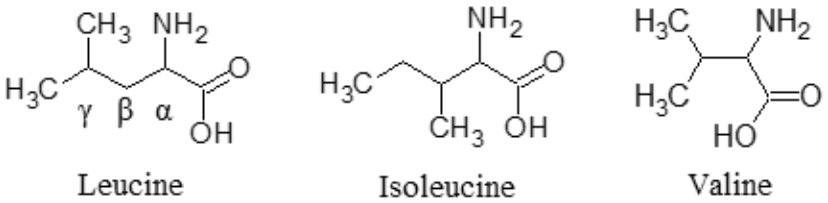


Figure 1.1. Molecular structure of the three branched-chain amino acids.

Animals require relatively large amounts of BCAA in their diets because skeletal muscle protein is composed of about 20% BCAA (Wilkinson et al., 2014). Fortunately, many food proteins are good sources of BCAA. Whey protein, for example, which is an important source of protein for neonates, is 23% BCAA, while casein contains 21% (National Research Council,

2012). Although lower in total protein, even plant proteins supply a fair amount of BCAA, about 17% in soybean meal and 20% in corn (National Research Council, 2012). BCAA are generally consumed in excess of their requirement, and those which are not incorporated into proteins are quickly catabolized (Harris et al., 2005).

Metabolism

Dietary BCAA come from either food proteins or crystalline amino acid supplements. Food proteins are broken down to di- and tri-peptides and amino acids by proteases before being transported into enterocytes by semi-specific transport proteins. The gastrointestinal tract utilizes an abundance of BCAA for energy and protein synthesis, with most amino acids utilized originating from arterial blood as opposed to first pass metabolism of consumed BCAA (Stoll & Burrin, 2006). About 40% of dietary and arterial BCAA combined are utilized by the gastrointestinal tract by the time they move to the liver via the portal vein (Stoll et al., 1998). The liver, on the other hand, removes only about 1% of BCAA during their initial pass (Hanigan et al., 2004) because it does not express the transaminase which initiates BCAA catabolism (Hutson et al., 1992; Sweatt et al., 2004). Dietary BCAA pass through the liver to arterial circulation, making them available to peripheral tissues. Skeletal muscles use and retain BCAA by incorporating them into proteins. Although the BCAA transaminase which initiates their catabolism is expressed in various tissues, its expression and activity are highest in skeletal muscle in humans (Suryawan et al., 1998). Following transamination in skeletal muscle, BCAA carbon returns to the bloodstream as branched-chain α-keto acids (BCKA) which are taken up and oxidized by the liver. BCAA nitrogen returns to the liver primarily as glutamine and alanine (Darmaun & Dechelotte, 1991). Nitrogen from BCAA is also used to synthesize nonessential

amino acids glutamate and aspartate (Holeček, 2021). The liver ultimately disposes of amino nitrogen via the urea cycle.

The first enzyme involved in catabolism of BCAA removes of the amino group via transamination to α-ketoglutarate. The enzyme branched-chain amino acid transaminase (BCAT) facilitates the transamination reaction between each of the three BCAA and α-ketoglutarate, producing glutamate and the BCKA corresponding to each BCAA. The BCKA produced from leucine is α-ketoisocaproate (KIC), α-ketomethylvalerat (KMV) is produced from isoleucine, and α-ketoisovalerate (KIV) corresponds to valine. However, the BCAA transamination reaction is reversible (Harris et al., 2005). There are two isoforms of BCAT, one which is located in the cytosol (BCAT1), and a mitochondrial isoform (BCAT2). The cytosolic isoform is primarily consolidated to the central nervous system, while BCAT2 is present in many tissues (Hutson et al., 1992; Sweatt et al., 2004).

All three BCKA are oxidized to branched-chain acyl-CoAs by the mitochondrial enzyme complex branched-chain keto acid dehydrogenase (BCKDH), also generating CO_2 and NADH. This reaction is irreversible and commits the BCAA metabolites to degradation. The BCKDH catalyzed reaction is also significant because it is the rate limiting step of BCAA catabolism, and BCKDH activity is highly regulated. Negative feedback is initiated by high ratios of NADH/NAD+ and acetyl-CoA/CoA (Brosnan & Brosnan, 2013). Additionally, BCKDH is inhibited by phosphorylation by BCKDH kinase (BCKDK). The substrates for BCKDH, particularly KIC, upregulate BCKDH by inhibiting BCKDK. Increased BCAA consumption increases BCKDH expression in the liver, but not in skeletal muscle (Wessels et al., 2016). Because the three BCAA share catabolic enzymes, supplementing one amino acid without the others can increase catabolism of the other two as well. For example, piglets supplemented with

leucine parenterally (Escobar et al., 2005), or in the diet (Manjarín et al., 2018; Wessels et al., 2016) had decreased plasma isoleucine and valine concentrations. Therefore, it is a good practice to supplement all three BCAA in order to prevent this issue (Manjarín et al., 2018). Similarly, because leucine potentially activates protein synthesis, plasma concentrations of most essential amino acids have been observed to decrease in piglets supplemented with leucine (Wilson et al., 2010). As with any growth promoter, amino acid consumption must be increased to provide material for newly synthesized proteins.

Branched-chain acyl-CoAs produced by BCKDH are committed to oxidation to acetyl-CoA and/or propionyl-CoA by a series of different enzymes (Figure 1.2). Leucine is purely ketogenic, generating acetyl-CoA and acetoacetate, whereas valine is glucogenic, generating propionyl-CoA, and isoleucine produces both. The terminal process in complete oxidation of BCAA carbon to CO_2 is the tri-carboxylic acid (TCA) cycle (Figure 1.3). Acetyl-CoA enters the TCA cycle where citrate synthase transfers the acetyl carbons to oxaloacetate to form the six-carbon citrate molecule. Acetyl-CoA fuels one complete turn of the TCA cycle, which allows two carbons to leave as CO_2, generates one GTP, and reduces three NADH and one $FADH_2$. When the reduced NADH and $FADH_2$ transfer their energy to the electron transport chain, one acetyl-CoA can generate a maximum of 12 ATP. When the reducing equivalents generated in earlier oxidation of KIC are included, one molecule of leucine can produce up to 39 ATP, superior to the maximum 36 ATP generated by one glucose molecule. Propionyl-CoA produced from KMV and KIV must be converted to succinyl-CoA before entering the TCA cycle. Propionyl-CoA is first carboxylated by propionyl-CoA carboxylase in an ATP dependent reaction and is isomerized by two more enzymes to form succinyl-CoA (Figure 1.2). Succinyl-

CoA provides positive net carbon to the TCA cycle, potentially contributing carbon to gluconeogenesis.

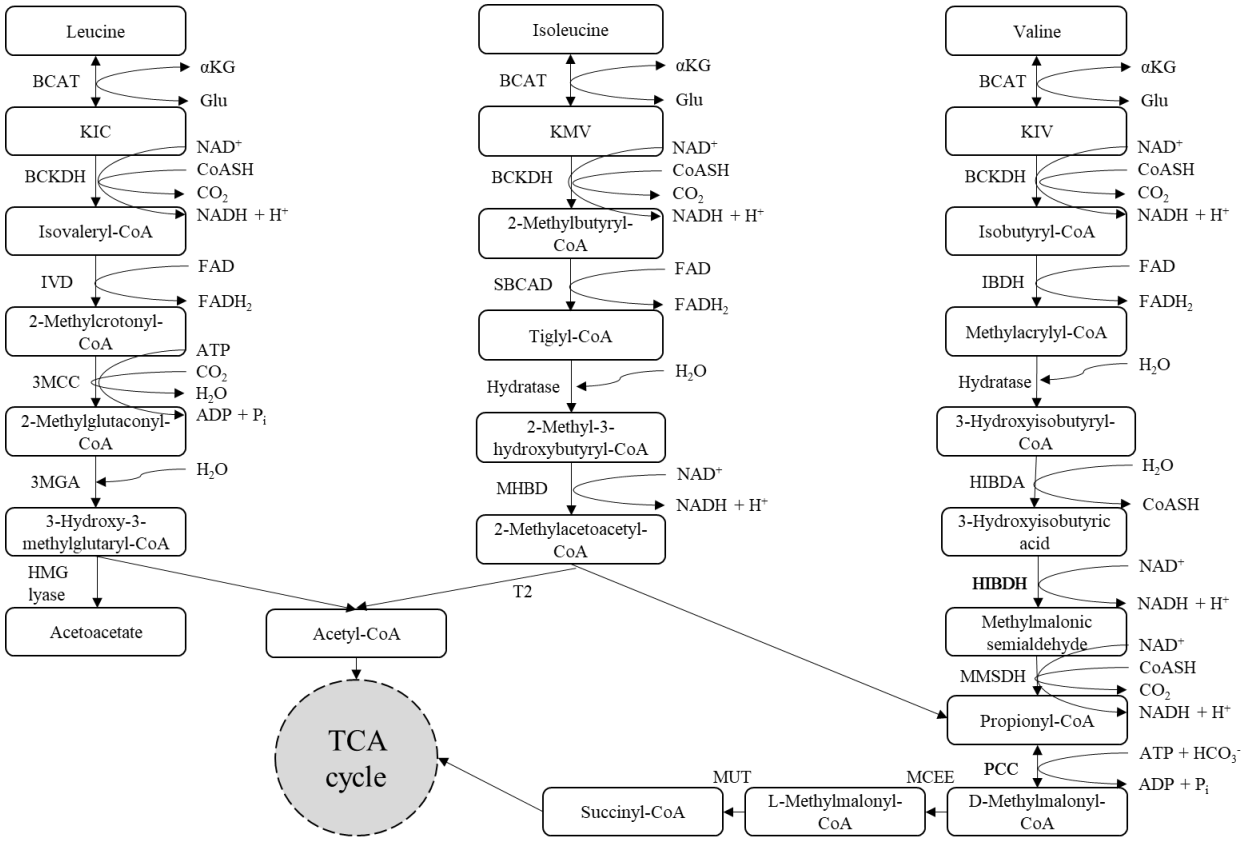


Figure 1.2. Catabolism of the branched-chain amino acids. KIC, α-ketoisocaproate; KMV, α-keto-β-methylvalerate; KIV, α-ketoivalerate; αKG, α-ketoglutarate; BCAT, branched-chain amino acid aminotransferase; BCKDH, branched-chain α-keto acid dehydrogenase complex; IVD, isovaleryl-CoA dehydrogenase; 3MCC, β-methylcrotonyl-CoA carboxylase; 3MGA, β-methylglutaconyl-CoA hydratase; SBCAD, acyl-CoA dehydrogenase; MHBD, β-hydroxyacyl-CoA dehydrogenase; T2, acetyl-CoA acyl transferase; IBDH, α-methyl acyl-CoA dehydrogenase; HIBDA, β-hydroxyisobutyryl-CoA hydrolase; HIBDH, β-hydroxyisobutyrate dehydrogenase; MMSDH, methylmalonic semialdehyde dehydrogenase; PCC, propionyl-CoA carboxylase; MCEE, methylmalonyl-CoA racemase; MUT, methylmalonyl-CoA mutase.

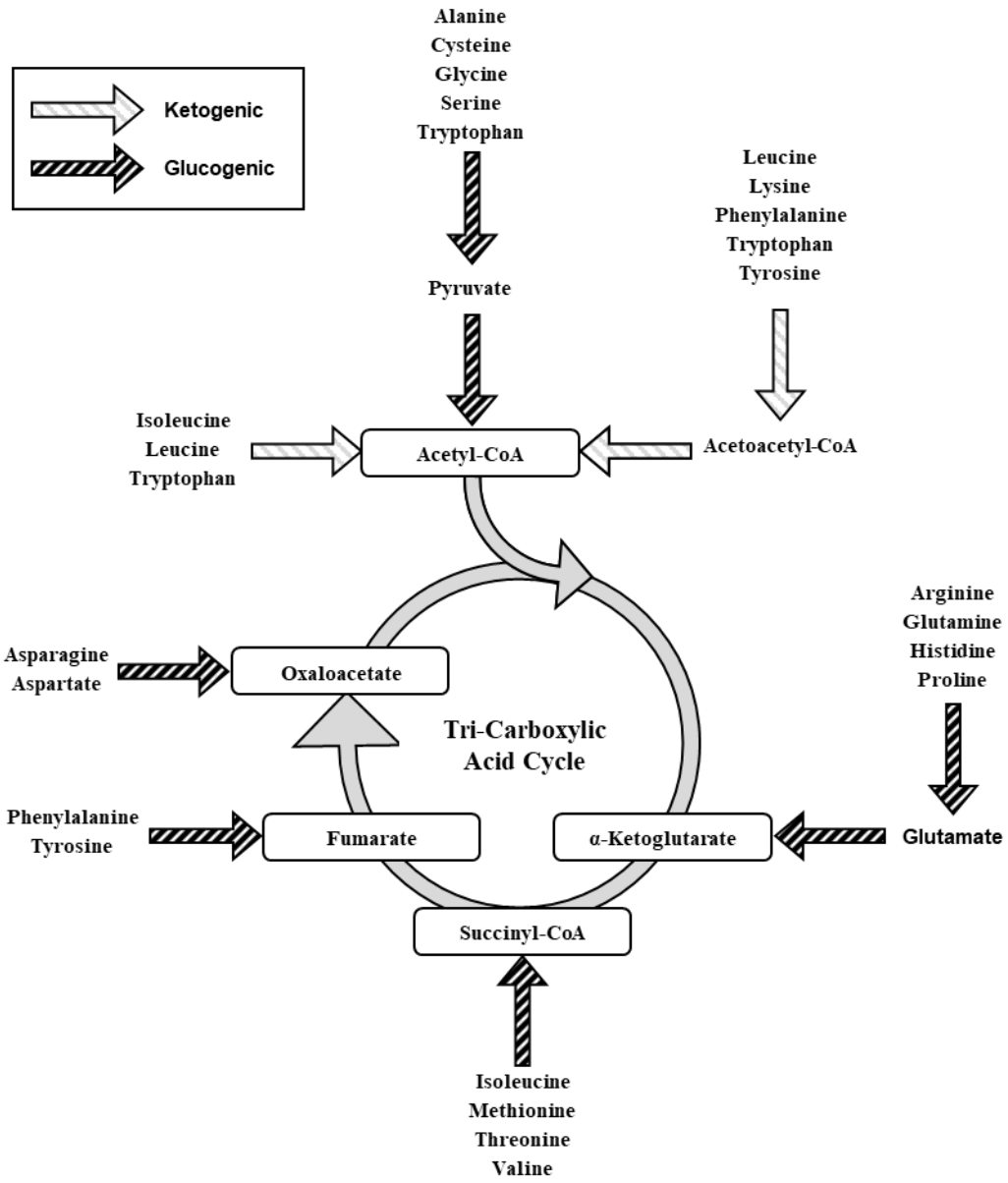


Figure 1.3. Amino acid entry points to the tri-carboxylic acid cycle. Leucine is strictly ketogenic, isoleucine is both ketogenic and glucogenic, and valine is strictly glucogenic.

Muscle Hypertrophy

Overiew

Attempts to increase muscle mass in low birth weight infants begin with identifying points of regulation in muscle hypertrophy. The mechanisms of muscle hypertrophy are well studied and remain an important area of research because of the importance of muscle in human health and animal meat production. In all animal species, skeletal muscles perform essential functions. They not only generate force and motion through coordinated contraction and relaxation, but skeletal muscle also plays essential roles in nutrient metabolism. Skeletal muscle consumes a large amount of energy, primarily by contracting and by synthesizing large amounts of contractile proteins to maintain function. Contractile proteins are also the only form of amino acid storage in the body. Therefore, skeletal muscle is responsible for amino acid supplies in response to the body's needs during severe periods of starvation.

Skeletal muscles increase in size by either developing additional muscle fibers, termed hyperplasia, or by increasing the size of existing fibers, termed hypertrophy. However, hyperplasia only occurs prenatally or in response to damaged fibers (Glass, 2003). Postnatally, muscle growth is driven by fiber hypertrophy (R. B. White et al., 2010). Muscle fiber hypertrophy requires protein accretion, which occurs when the rate of protein synthesis exceeds the rate of protein degradation in the cell (Miyazaki & Esser, 2009). Muscle growth is more rapid during the neonatal period than at any other time in an animal's life (Reeds et al., 2000). The rate of myofibrillar protein synthesis is highest, and their rate of degradation lowest, at this time, but the difference between the two quickly narrows with age (Davis et al., 1996; Fiorotto et al., 2000). In other words, neonates utilize dietary amino acids much more efficiently than adults

(D. G. Burrin et al., 1992). Decreased protein synthesis with aging is largely due to decreased number of ribosomes in muscle fibers (Davis et al., 2001). However, neonates rely on multiple mechanism to achieve maximal protein synthesis.

One way for a muscle fiber to increase protein synthesis is to increase protein synthetic capacity. The central dogma of molecular biology states simply that DNA contains the genetic templates which are transcribed to mRNA, which is ultimately translated to proteins. Therefore, increasing cellular DNA content is the first step towards increasing the amount of protein that cell can synthesize. In fact, muscle protein accretion has been shown to be preceded by increased DNA content in growing pigs (Harbison et al., 1976; Swatland, 1977). Muscle fibers are multinucleated cells, which retain the capacity to incorporate more nuclei throughout life (Moss & Leblond, 1971). Up to 50 to 90% of DNA in skeletal muscle is accumulated after birth (Allen et al., 1979). Additional myofibrillar nuclei are gained by the incorporation of satellite cells (Rhoads et al., 2009; Wozniak et al., 2005). Satellite cells are muscle-specific adult stem cells. A population of satellite cells reside in skeletal muscles adjacent to muscle fibers, between the sarcolemma and basal lamina (Murach et al., 2018). They reside in a quiescent state until activated by a stimulus from growth, injury, or mechanical stress when they begin to replicate, and some are incorporated into muscle fibers which use their DNA to produce muscle proteins (Relaix & Zammit, 2012). Satellite cells are most active in young animals. Newborn animals also maintain a larger pool of satellite cells than adults, decreasing from 30% (Allen et al., 1979) of myonuclei to 6% in adulthood (Snow, 1977). Mature animals have lower rates of protein turnover and mostly only require maintenance of muscle mass. At this point, there is evidence in mice that satellite cells are no longer necessary to maintain muscle mass (Keefe et al., 2015; Murach et al., 2018).

Another way to increase protein synthesis is to increase protein synthetic efficiency. In terms of the central dogma, that is to increase the rate of translation of mRNA to protein. The required materials of translation include mRNA, amino acid charged tRNA, energy in the form of ATP, and all the subunits of a ribosome (Preiss & Hentze, 2003). For this reason, adequate nutrition is required to supply amino acids and energy for protein synthesis and muscle growth. Both protein (Kao et al., 2016) and energy (Hector et al., 2018) deficiency decrease rate of protein synthesis. The body also has nutrient detecting mechanisms which allow it to regulate metabolism according to nutrient availability.

Molecular Mechanisms of Protein Synthesis

Protein synthesis relies on mRNA translation which consists of three phases: initiation, elongation, and termination (Sonenberg & Hinnebusch, 2009). Of these, initiation is rate-limiting and highly regulated. The two primarily regulated steps of translation initiation are 1) the recruitment of methionyl tRNA to the 40S ribosomal subunit to form the 43S preinitiation complex, which is not acutely affected by nutrient intake (Davis et al., 2000), and 2) binding of mRNA to the eIF4F complex, which is strongly activated following a meal (Vary & Lynch, 2006). Recruitment of methionyl tRNA is accomplished by eukaryotic initiation factor 2 (eIF2), which is GTP dependent (Schmitt et al., 2010). eIF2B recharges eIF2 with GTP, which is the cite of regulation by phosphorylation by eIF2α (Kimball, 1999). For the preinitiation complex to bind mRNA, the eIF4F complex must be assembled from eIF4E, eIF4G, and eIF4A (Jackson et al., 2010). This step is directly inhibited by eIF4E binding protein 1 (4EBP1), which forms a complex with eIF4E, thereby preventing it from binding with eIF4G. Once these two steps are complete, the 43S subunit and eIF4F form the 48S preinitiation complex (Gingras et al., 2001; N.

Hay & Sonenberg, 2004). The 48S complex scans for the AUG codon, and upon recognizing it, GTP-eIF2 is hydrolyzed to GDP-eIF2, and it dissociates from the complex along with most of the other initiation factors (Sonenberg & Hinnebusch, 2009). This allows the preinitiation complex to bind with the large 60S subunit, forming the functional 80S initiation complex capable of elongation (Pestova et al., 2007).

The central hub connecting nutrient and growth factor signals to changes in translation initiation is the kinase mechanistic target of rapamycin (mTOR) (Weigl, 2012). mTOR forms a complex with six proteins including regulatory associated protein of mTOR (raptor), known as mTORC1, which directly regulates protein synthesis in animals (Jacinto & Hall, 2003), and is potently inhibited by the drug rapamycin. A similar complex, mTORC2 is composed of seven proteins including mTOR as well as rapamycin-independent companion of mTOR (rictor), and stress-activated protein kinase-interacting protein 1 (SIN1). Opposed to mTORC1, mTORC2 is not acutely inhibited by rapamycin and does not directly affect protein synthesis (Laplante & Sabatini, 2012). Rather, mTORC2 phosphorylates Akt in response to growth factors, functioning upstream of mTORC1 and affecting cell metabolism and survival (Laplante & Sabatini, 2012).

The main downstream effectors of mTORC1 regulate translation initiation. mTORC1 directly phosphorylates 4EBP1, preventing it from binding eIF4E and allowing formation of the eIF4F complex (Gingras et al., 2001; N. Hay & Sonenberg, 2004). The other major target of mTORC1 phosphorylation is ribosomal protein S6 kinase beta-1 (S6K1). mTORC1 phosphorylates S6K1 at Thr389, which allows docking of phosphoinositide-dependent protein kinase-1 (PDK1), which subsequently activates S6K1 by phosphorylating at Thr229 (Keshwani et al., 2009). Phosphorylation of S6K1 by mTORC1 requires scaffolding from the protein eIF3 (Csibi et al., 2010; Holtz et al., 2005). Once activated, S6K1 activates translation initiation by

phosphorylating eIF4B and programmed cell death 4 (PDCD4), preventing it from inhibiting eIF4A from joining eIF4F complex (Dennis et al., 2012).

Molecular Mechanisms of Protein Degradation

As previously mentioned, muscle fiber hypertrophy results from protein accretion when rates of protein synthesis exceed rates of degradation (Miyazaki & Esser, 2009). Therefore, protein accretion can occur even when protein degradation is high, as long as protein synthesis is greater. This is the case, for example, when protein turnover is high in rapidly growing young animals (Fiorotto et al., 2000). Protein degradation is continuous process required to replace old and misfolded proteins and provide free amino acids as needed (A. L. Goldberg, 2003; Lecker et al., 2006). Three different protein degradation pathways serve separate roles in protein breakdown which contributes to protein turnover in healthy cells. These systems are the autophagy-lysosome, the calpain, and the ubiquitin-proteosome systems (Ventadour & Attaix, 2006). A fourth proteolytic system, carried out by the caspase family of proteins, is essential for apoptosis in diseased or damaged tissue, but plays no known role in protein turnover in healthy cells (Shalini et al., 2015)

Autophagy-lysosome

The autophagy-lysosome functions to degrade relatively large cellular components including whole organelles and proteins to their basic materials for recycling of nutrients (Sandri, 2011). In this way, it is responsible for regulating half-life of organelles and proteins (Mizushima et al., 2008). Lysosomes are membrane-bound vesicles containing a low pH interior with various acidic hydrolases (Bechet et al., 2005). There are three different routes leading a protein or organelle to lysosomal degradation. The first is microautophagy, in which the

lysosome directly engulfs a portion of cytoplasm and its contents (Mijaljica et al., 2011). Another route is chaperone-mediated autophagy, in which hsc70 chaperone protein interacts with both the target protein and a lysosomal membrane protein, effectively delivering proteins to the site of degradation (Cuervo & Dice, 1996). Lastly, in macroautophagy, proteins and organelles are enveloped by double membraned vesicles called autophagosomes, which then fuse with lysosomes. Macroautophagy is the most studied route in skeletal muscle (Bonaldo & Sandri, 2013). Lysosomes become a rich source of nutrients for cells. Depending on an organisms metabolic requirements, lysosomal nutrients can be used for energy during starvation or for protein synthesis (Kaur & Debnath, 2015; Kuma et al., 2004).

Calpain

The calpain system consists of two calcium-activated proteases, μ-calpain and m-calpain, and their regulatory polypeptide calpastatin (Goll et al., 2003). The two proteases are named for the concentration of calcium at which they are activated: μM or mM. Neither calpains degrade actin or myosin, but both calpains release α-tubulin from the Z-disc by degrading other myofibrillar proteins including α-actinin, troponin I and T, tropomyosin, desmin, nebulin, titin, filamin, C-protein, dystrophin, and M-protein (Goll et al., 1991) as well as cytoskeletal proteins viniculin, spectrin, and talin, (Goll et al., 1992). Despite our understanding of how calpains work, relatively little is known about their function in living tissue. Based on what is known about calpains, they are essential for releasing large myofibrillar proteins so that they may be degraded by the ubiquitin-proteasome system (Roos-Mattjus & Sistonen, 2004). Inhibition of calpains in myoblast cells reduced protein degradation by 30% (Huang & Forsberg, 1998). Expression of calpain mRNA is lowest in neonatal pigs but increases with age, suggesting

calpains play a role in the rapid muscle accretion of young animals (Z. Li et al., 2009). Additionally, calpains play a critical and well characterized role in meat tenderization postmortem (Wicks et al., 2019).

Ubiquitin-Proteosome

The ubiquitin-proteosome system degrades the majority of intracellular proteins (Rock et al., 1994), including sarcomeric proteins (Lecker et al., 2006), to peptides which are subsequently degraded by intracellular peptidases to individual amino acids. Ubiquitin is a polypeptide cofactor which marks proteins for degradation (Glickman & Ciechanover, 2002). Three types of enzymes are required to attach ubiquitin to a protein: E1s are ubiquitin-activating enzymes, E2s are ubiquitin-carrier or conjugating proteins, and E3s are ubiquitin-protein ligases. E3 ligases are of particular importance because they are the rate limiting step in the degradation pathway and are nutritionally regulated (Lecker et al., 2006). Old or defective proteins are "tagged" for degradation with the covalent addition of small ubiquitin peptides which accumulate in a chain, called polyubiquitination. Once polyubiquitinated, the target protein docks with the 26S proteasome and is fully degraded to amino acids.

RING finger-1 protein (MuRF1) and Atrogin-1 are muscle-specific E3 ligases which are of importance to muscle protein degradation. Transcription of the Atrogin-1 gene is regulated by the forkhead box proteins (FOXO) family of transcription factors, which are targets of Akt (Weigl, 2012). When phosphorylated by Akt, FOXO cannot enter the nucleus to promote Atrogin-1 transcription (McLoughlin et al., 2009). MuRF1 transcription is also decreased by Akt phosphorylation, although the pathway is not fully understood (Stitt et al., 2004). The ubiquitin-proteosome system is the only protein degradation pathway shown to be regulated by insulin.

Atrogin-1 and MuRF1 expression are also decreased by branched-chain amino acids (Borgenvik et al., 2012). On the other hand, their expression in skeletal muscle are increased during exercise to aid protein turnover (Borgenvik et al., 2012).

Activation Of mTOR

Factors which influence protein synthesis have been investigated since the 1970s when methods were developed to measure protein synthesis and degradation of isolated tissues in vitro (Garlick, 2005). In the first of such experiments to examine skeletal muscle, insulin and amino acids increased protein synthesis and decreased degradation in rat diaphragm muscle, while glucose, octanoate, and beta-hydroxybutyrate did not (Fulks et al., 1975). Furthermore, that early study identified a BCAA mixture to be the only amino acids responsible for initiating protein synthesis in skeletal muscle. Later, an in vivo experiment showed that postabsorptive rats receiving intravenous insulin had increased muscle protein synthesis, although protein synthesis was higher still in rats fed a full meal (Garlick et al., 1983). In addition to demonstrating the anabolic effect of insulin in vivo, the authors concluded that other nutritional factors are required to initiate protein synthesis. When BCAA were infused along with glucose, rate of protein synthesis increased beyond that resulting from insulin infusion and equal to a complete mix of amino acids plus glucose (Garlick & Grant, 1988). It is now understood that protein synthesis is coupled to environmental stimuli by mTORC1. Insulin and amino acids represent nutrient availability and trigger protein synthesis as a means of amino acid utilization. Insulin-like growth factor 1 (IGF-I) stimulates protein synthesis through mTORC1 activation in response to specific stages of development and adequate plane of nutrition.

Insulin and IGF-I

Insulin is a peptide hormone which plays a central role in nutrient metabolism. The most widely recognized function of insulin is to stimulate glucose uptake and storage by glycogenesis in cells throughout the body. Insulin is secreted by pancreatic beta cells in response to increased plasma glucose concentration, as results from carbohydrate consumption. Glucose is taken up by β-cells and metabolized, generating ATP which causes ATP gated K^+ channels to close, resulting in depolarization of the cell. Depolarization causes voltage-gated Ca^{2+} channels to open, and increased Ca^{2+} concentration initiates exocytosis of insulin-containing vesicles into the blood (Rorsman & Braun, 2013).

Insulin is also considered an anabolic hormone because it upregulates the synthesis and accumulation of carbohydrates, lipids, and protein in various tissues while inhibiting catabolic reactions (Rhoads et al., 2016). Insulin stimulated protein synthesis in skeletal and cardiac muscle in fasted rats and pigs, but did not have the same effect in visceral tissues (Bark et al., 1998; Davis et al., 2001). The insulin receptor is a tyrosine kinase receptor. When insulin or IGF-I bind with the insulin receptor, the receptor autophosphorylates, leading to recruitment and phosphorylation of insulin receptor substrate proteins (IRS) at tyrosine residues. Activated IRS mediates intracellular responses. The main signaling pathway initiated is the phosphatidylinositol 3-kinase (P13K)-Akt pathway (Weigl, 2012). This pathway is propagated by activation of P13K by phosphorylated IRS. P13K then phosphorylates plasma membrane-associated phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol-3,4,5-triphosphate (PIP_3). PIP_3 provides a docking site for phosphoinositide-dependent protein kinase (PDK) to phosphorylate and activate Akt. The kinase activity of Akt is responsible for various metabolic actions of glucose, including, but not limited to, translocation of glucose transporter 4 (GLUT4)

for glucose uptake, mTOR activation, and inhibition of proteolytic ubiquitinases (Weigl, 2012). Akt activates mTORC1 by inhibiting inhibitory proteins including tuberous sclerosis complex 2 (TSC2) and proline-rich Akt substrate of 40 kDa (PRAS40) (Huang & Manning, 2009; H. Wang et al., 2012). In addition to initiating protein synthesis, insulin and IGF-I have the power to stimulate proliferation of satellite cells (Cassar-Malek et al., 1999; Gonzalez et al., 2020; Illario et al., 2009).

IGF-I is a peptide hormone similar in structure to insulin (Duan et al., 2010). IGF-1 and insulin share similar intracellular mechanisms of mTOR activation, and even activate each other's receptors. The major difference between the two are the distinct physiological functions for which they provide anabolic signaling. While insulin signals acute energy availability, IGF-I signals developmental need for growth and overall plane of nutrition (Oksbjerg et al., 2004). While insulin is produced in pancreatic β-cells and acts in an endocrine manner, IGF-1 is produced by many tissues and cell types including skeletal muscle, and acts as an autocrine, paracrine, and endocrine hormone. The endocrine function of IGF-I was discovered before the others and termed the "somatomedin hypothesis" (Kaplan & Cohen, 2007). In this model, growth hormone stimulates IGF-I production in the liver, and the liver secretes IGF-I into general circulation to affect other tissues. While this hypothesis is not incorrect, autocrine and paracrine effects have since been discovered (D'Ercole et al., 1984). In fact, the locally produced IGF-I was found to me more important than hepatic IGF-I for muscle hypertrophy (Yakar et al., 1999). The importance of local IGF-I for muscle growth in neonates is demonstrated by high mRNA expression of IGF1 and its receptor in skeletal muscle of neonatal pigs, which declines with age (Gerrard et al., 1998). Furthermore, IGF binding proteins 3, 4, and 5 expression similarly decrease in skeletal muscle from birth to maturity (Clemmons, 2009).

This is in contrast to circulating IGF-I, which peaks in adulthood when growth has effectively concluded (Puche & Castilla-Cortázar, 2012).

Amino Acids

Sensing nutrient availability enables organisms to seize the opportunity for growth, and to store or oxidize excess nutrients for energy. While the mechanisms for sensing and responding to blood glucose are relatively well understood, mechanisms of detecting amino acids are a very current area of research. During amino acid starvation, the general control non-derepressible pathway inhibits protein synthesis in response to uncharged tRNA concentration (Taylor, 2014). Additionally, amino acid transport proteins at the cellular and lysosomal membranes may sense and regulate cytosolic amino acid concentrations (Taylor, 2014). Finally, amino acid availability is established to initiate translation initiation via the mTOR pathway. Orally administered leucine upregulated translation initiation in rats by increasing phosphorylation of 4EBP1, allowing formation of the eIF4F complex (Anthony, Anthony, et al., 2000). Shortly thereafter, rapamycin was used to demonstrate that initiation of translation by leucine is mTOR dependent (Anthony, Yoshizawa, et al., 2000). Although leucine activates mTOR and its downstream effectors similarly to insulin and IGF-I, leucine has been shown to work independently of insulin (Anthony, Yoshizawa, et al., 2000; Crozier et al., 2005; Escobar et al., 2006). The amino acid sensing pathways upstream of mTOR are still being identified, and recent research has uncovered sensors of arginine and leucine.

Amino acid activation of mTOR requires docking of mTORC1 at the lysosomal membrane. Lysosomes are a rich source of free amino acids because they function to degrade and recycle cellular proteins. Multiple amino acid transport proteins reside at lysosome

membranes (Dickinson & Rasmussen, 2013). Docking of mTORC1 at the lysosomal membrane is mediated by Ras-related (Rag) GTPases (Kim et al., 2008). Rag A forms a heterodimer with Rag B, and Rag C forms a heterodimer with Rag D. In the active conformation, Rag A/B-GTP and Rag C/D-GDP associate with the lysosomal membrane-bound Ragulator protein complex (Bar-Peled et al., 2012; Sancak et al., 2008). The Rag proteins bind the RAPTOR component of mTORC1 to anchor the complex at the lysosomal membrane.

Cytosolic amino acid concentration regulates mTORC1 activity through the Rag GTPases. Leucine activates mTORC1 by binding directly to, and inhibiting, two cytosolic proteins with highly similar functions: Sestrin2 and to SAR1B (Vellai, 2021). This function of Sestrin2 was only identified around 2016 (Wolfson et al., 2016) and SAR1B in 2021 (J. Chen et al., 2021). When not bound to leucine, Sestrin2 and SAR1B inhibit mTORC1 activation by binding the GATOR2 complex, at different subunits (Vellai, 2021). When GATOR2 is prevented from inhibiting GATOR1, GATOR1 inhibits the Rag GTPases. In this state, mTOR is not able to dock at the lysosome membrane, and it remains inactive. Similarly, arginine inhibits the protein CASTOR1 (Figure 1.4), which otherwise inhibits GATOR2 and inhibits mTORC1 activation by the same pathway as Sestrin2 and SAR1B (Chantranupong et al., 2016).

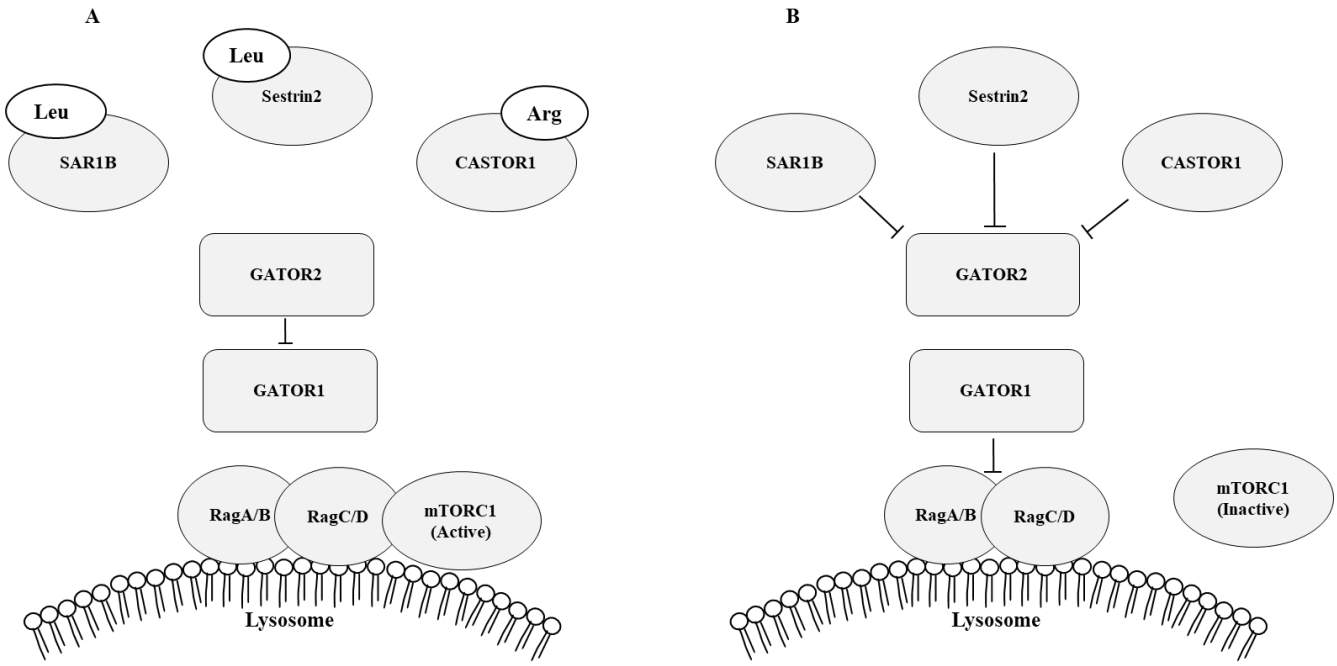


Figure 1.4. Cytosolic pathway of amino acid activation of mTOR. A) Amino acid concentrations are high enough that leucine binds to Sestrin2 and SAR1B, and arginine binds to CASTOR1, allowing the GATOR2 complex to inhibit the GATOR1 complex from preventing mTORC1 from anchoring to the Rag proteins at the lysosomal membrane. Subsequently, mTORC1 anchors at the lysosomal membrane and can propagate the signal for protein synthesis. B) Leucine and arginine concentrations are not high enough to inhibit Sestrin2, SAR1B, or CASTOR1, allowing these proteins to inhibit GATOR2. Without inhibition from GATOR2, GATOR1 prevents the Rag proteins from recruiting mTORC1 to the lysosome, and mTORC1 remains inactive.

In piglets, leucine supplementation to low protein diets decreases formation of Sestrin2-GATOR2 complex, and increased RagA-mTOR and RagC-mTOR complexes (Suryawan et al., 2017). Leucine is repeatedly demonstrated to activate mTOR either by western blots of phosphorylated mTOR (Suryawan et al., 2008) or phosphorylated S6K1 and 4EBP1 (Escobar et al., 2005) in numerous species including humans (Drummond & Rasmussen, 2008). Isoleucine and valine did not have the same effect on mTOR in skeletal muscle of rats or pigs (Anthony, Yoshizawa, et al., 2000; Escobar et al., 2006), but the leucine metabolite β-hydroxy-β-methylbutyrate increased protein synthesis to the same degree as a high protein diet in neonatal

pigs (Kao et al., 2016). mTOR responds differently to amino acids according to tissue type. Leucine activates mTOR in most tissues, excluding the liver (Murgas Torrazza et al., 2010). Additionally, isoleucine, methionine, and threonine also increased phosphorylation of mTOR in rat mammary tissue (G. M. Liu et al., 2017).

Supplementation of Branched-Chain Amino Acids

The function of leucine to signal initiation of protein synthesis leads to the question as to whether it can enhance skeletal muscle hypertrophy. Populations who could benefit from enhanced muscle growth include those with impaired muscle growth, muscle wasting, or the desire to increase muscle mass for athletic performance. Specifically, individuals born with low birth weight have impaired muscle growth (Brown & Hay, 2016), elderly people are prone to sarcopenia (Trombetti et al., 2016), and many people look to build muscle by resistance training. However, the extent of leucine's therapeutic value remains debatable.

Most people are aware of BCAA because they have seen supplements on grocery store shelves. Labels on supplements promise increased muscle mass. Indeed, it is encouraging that leucine causes activation of mTOR and muscle protein synthesis in postabsorptive rats (Anthony, Yoshizawa, et al., 2000; Crozier et al., 2005) and neonatal pigs (Escobar et al., 2006). However, evidence of leucine stimulated protein synthesis in postabsorptive humans is lacking (Wolfe, 2017). The few investigations into this found that leucine infusions had no effect on muscle protein synthesis in fasted adult subjects, but they did decrease muscle protein breakdown (Louard et al., 1990, 1995). Leucine can decrease muscle protein degradation by suppressing the autophagy-lysosome system, as demonstrated in neonatal pigs (Boutry et al., 2013).

It is recommended to consume BCAA supplements following resistance training. Resistance training increases protein turnover, in other words, both rates of muscle protein synthesis and protein breakdown, in fasted subjects, but ultimately causes a net increase in protein balance (Phillips et al., 1997, 1999). At the molecular level, resistance training activates mTOR signaling in skeletal muscle without nutritional input, through an Akt independent mechanism (Eliasson et al., 2006). In this way, the effect of exercise on initiating protein synthesis is very similar to the effect of leucine. However, the effect of resistance training and leucine can be additive. Young men consuming a BCAA supplement without additional nutrients had increased mTOR activation and muscle protein synthesis after a session of resistance training than men who consumed a placebo supplement (Jackman et al., 2017). Although leucine contributes to maximal protein synthesis after resistance training, the same effect can be achieved from a quality complete protein like whey protein (Tipton et al., 2009). When taking a BCAA supplement, it is important to consider that initiation of protein synthesis does not necessarily result in significant muscle mass accretion unless materials and energy to support protein synthesis are sufficiently available. For example, leucine infusion increased muscle protein synthesis in rats for about 30 minutes before returning to baseline, whereas a mix of essential amino acids sustained protein synthesis through the four hour duration of infusion (Kobayashi et al., 2006). Similarly, leucine infusion did not increase protein synthesis compared to saline in neonatal pigs unless essential amino acids were included (Escobar et al., 2007; Wilson et al., 2010)

Loss of muscle mass is inevitable with aging, and causes mobility and quality of life concerns among the elderly (Trombetti et al., 2016). Leucine is a potential treatment to preserve muscle mass based on its anabolic properties. There have been a couple encouraging studies, but

to this point there are too few controlled trials to draw any conclusions (Plotkin et al., 2021). A recent controlled trial showed no benefit of leucine supplementation to elderly participants on muscle mass or strength (Murphy et al., 2021).

Finally, individuals affected by LBWT are born with relatively small muscle mass, and their muscles grow at a slower rate than individuals born of normal birth weight (Brown & Hay, 2016). The origins of LBWT and characterization of their muscle mass will be detailed in the following chapter. Their deficits in muscle growth, however, make them a population which can benefit from increased muscle hypertrophy. Another consideration for LBWT infants is that their underdeveloped digestive systems may not be able to tolerate normal meal feeding. Being restricted to continuous feeding via gastric intubation has a detrimental effect on muscle growth because meal feeding, or even intragastric meals delivered in intermittent boluses, greatly enhance anabolic signaling and protein synthesis compared to continuous feeding in neonatal pigs (El-Kadi et al., 2012). During continuous feeding, parenteral leucine pulses improved muscle protein synthesis closer to intermittent feeding in neonatal pigs (Boutry et al., 2013). In a similar experiment, leucine pulses increased protein synthesis during continuous feeding, while alanine pulses did not (Boutry et al., 2016). That study is also a rare example of leucine supplementation increasing lean gain. In a more conventional piglet population, suckling pigs orally supplemented leucine for two weeks gained more weight than those given alanine (Y. Sun et al., 2015). That study is limited, however, by not specifying whether or not the weight increase was lean tissue, and by the fact that leucine may be limiting in sow's milk (Rezaei et al., 2013). LBWT pigs may have a higher leucine requirement, as a dose-response trial demonstrated that lighter weaned pigs have a higher leucine requirement for average daily gain and feed conversion than heavier pigs (Bertocchi et al., 2019). Furthermore, leucine increased

anabolic signaling and average daily gain in weaned LBWT pigs fed *ad libitum* but not NBWT pigs (Xu et al., 2016).

There are also a couple examples of BCAA initiating protein synthesis in neonatal pigs without corresponding lean tissue accretion after 21 days of feeding. When leucine was added to low protein diets, translation initiation and muscle protein synthesis were increased, without improving lean gain (Columbus et al., 2015). Similarly, low protein, low energy diets supplemented with leucine did not affect protein synthesis or lean gain despite increasing translation initiation signaling (Manjarín et al., 2018). These studies provide further evidence that despite initiating anabolic processes, BCAA supplementation alone cannot increase lean tissue accretion without sufficient protein and energy to support protein synthesis. Whether leucine supplementation can improve lean growth in LBWT neonates consuming adequate nutrients remains a gap in knowledge which is addressed by the research in this dissertation.

Intrauterine Growth Restriction

A variety of factors can result in infants being born undersized and underdeveloped. When fetal growth is limited, the fetus is small for gestational age is affected by intrauterine growth restriction. Intrauterine growth restricted infants are born LBWT. It is estimated that 5 to 10% of infants in the world are born LBWT (Nardozza et al., 2017). Intrauterine growth restricted infants are the focus of this research, but LBWT infants can also result from premature birth, or simply be small yet healthy or adequate for gestational age. LBWT often results from either chromosomal disorders or from placental insufficiency, which is an inadequate supply of maternal nutrients, oxygen, or space for normal conceptus growth (Resnik, 2002). Placental insufficiency can result from a range of maternal conditions including but not limited to under-

or overnutrition, immature body size, infection, drug and alcohol intake, and smoking (Rosenberg, 2008; Wu et al., 2006). The risk of neonatal mortality is 2 to 4% higher for LBWT infants than those not affected by LBWT (Vayssière et al., 2015). LBWT causes delayed cognitive development and long-term consequences including obesity, coronary heart disease, hypertension, dyslipidemia, type 2 diabetes, and chronic lung and kidney diseases (Longo et al., 2013).

Much research has been done towards understanding, preventing, and treating LBWT. However, controlled trials in human infants and even nonhuman primates are impractical and unethical in many cases, so laboratory and agricultural animals are used instead. The optimal animal model for LBWT infants is the neonatal pig. LBWT occurs in pigs commonly and spontaneously (Ferenc et al., 2014). Alternatively, LBWT can be induced for research by premature delivery, maternal nutrient restriction, glucocorticoid dosing, or uterine artery ligation. Neonatal LBWT pigs are distinguished by asymmetrical growth patterns similar to LBWT human infants (R. Bauer et al., 2003). Pigs share very similar physiology and metabolism to human infants, making them suitable for nutrition trials (D. Burrin et al., 2020; Odle et al., 2014). Furthermore, LBWT pigs can be matched with genetically similar NBWT littermates to reduce genetic variation in experiments. LBWT pigs often result from restricted uterine space. Pig fetuses at the end of the uterine horns grow larger than those positioned between two littermates (Perry & Rowell, 1969). Modern hyper-prolific sows generate LBWT piglets more frequently. Large litter size increases variation in piglet birth weight, and LBWT pigs have slower growth rates and poorer and survival (Quiniou et al., 2002).

LBWT infants can be born with various underdeveloped organs which affect nutrient utilization, growth, and overall health. LBWT infants survive harsh intrauterine environments by developmental and metabolic adaptations. In order to make the most out of scarce nutrients, LBWT infants develop what is coined the "thrifty phenotype" (Hales & Barker, 1992). One consequence of the thrifty phenotype is asymmetrical fetal growth patterns (Lubchenco LO et al., 1966). Growth of the head is the top priority, and internal organs are prioritized over muscle and fat. This asymmetric growth pattern can be observed in the resulting infants with bulbous heads and low body fat. In addition to growth and metabolic disorders, studies in LBWT pigs and sheep have identified abnormal development and dysfunction in testes, ovaries, brain, heart, skeletal muscle, liver, thymus, small intestine, and mammary gland (Wu et al., 2006). Undersized liver, intestines and pancreas contribute to persistent issues in nutrient utilization (Ferenc et al., 2014). In addition, LBWT growing pigs had decreased digestibility of dry matter and energy, and decreased flux of glucose and triglycerides in the portal vein (T. Li et al., 2021).

Placental insufficiency disrupts myogenesis, permanently affecting skeletal muscle development. The first multinucleated muscle fiber precursor cells to form are called primary myotubes. Primary myotubes serve as a scaffold along which secondary myotubes form (Picard et al., 2002). Secondary myotubes develop into secondary muscle fibers, which greatly outnumber primary fibers (Dwyer et al., 1994; Ward & Stickland, 1991). LBWT animals are born with fewer muscle fibers (Greenwood et al., 2000; Nissen et al., 2003; Park et al., 2011; Rehfeldt & Kuhn, 2006). Decreased number of muscle fibers in LBWT animals is accounted for by fewer secondary fibers, while the number of primary fibers is similar to NBWT animals (Dwyer et al., 1994; Ward & Stickland, 1991). Muscle fiber number is fixed at birth (Glass,

2003; Miyazaki & Esser, 2009). Therefore, muscles with fewer fibers are a permanent detriment to lean growth in LBWT animals. Furthermore, LBWT pigs are also born with a smaller population of satellite cells than NBWT pigs (Stange et al., 2020). Muscle contraction and other metabolic functions in skeletal muscle consume a large portion of energy in the body. Insufficient muscle mass to remove blood glucose may contribute to LBWT individuals developing insulin resistance in adulthood (Hermann et al., 2003; Jensen et al., 2007).

Because LBWT individuals have a relatively small number of muscle fibers, it is important to make up for that muscle mass. The only way that can happen is through muscle fiber hypertrophy. Unfortunately, LBWT pigs have lower protein accretion than their NBWT littermates because protein synthesis is compromised, although protein degradation is not (Zhu et al., 2015). In a proteomics experiment, LBWT pigs expressed fewer protein synthetic enzymes and more degradation enzymes at birth (J. Wang et al., 2008). Decreased muscle protein synthesis in LBWT pigs has been traced back to translation initiation signaling. Although birth weight did not affect mTOR phosphorylation in skeletal muscle, S6K1 phosphorylation was still lower in newborn LBWT pig muscle, which could be due instead to lower eIF3 abundance, which serves as a scaffolding protein for S6K1 phosphorylation by mTOR (Y. Chen et al., 2016). Despite impaired muscle protein synthesis in LBWT pigs, they also have some compensatory changes. Neonatal LBWT pigs express less 4E-BP1, myostatin, myostatin receptor, and phosphorylated Smad3 than NBWT pigs, which would be advantageous for protein synthesis (Y. Chen et al., 2016). How long these compensatory mechanisms persist, and how they can be used to the animal's advantage are still unknown. Regardless, the neonatal period is

the most active time for muscle development, making it a critical time for intervention (Davis et al., 1996; Reeds et al., 2000).

The accelerated growth rate in response to restoration of adequate nutrition following nutrient restriction is referred to as compensatory gain. A classic example is when ad libitum feeding was restored to pigs who were feed restricted for 50 or 60 days after weaning, they had improved daily gain and feed efficiency compared to pigs fed ad libidum the entire time, and reached the same slaughter weight (Therkildsen et al., 2004). Similarly, children affected by LBWT experience compensatory gain during the first year of life (Beger et al., 2018; Soto et al., 2003), as do piglets (Myrie et al., 2011; K. R. Poore & Fowden, 2004). Despite accelerated growth rate, LBWT children do not necessarily reach the same height or weight as NBWT children (Fitzhardinge & Inwood, 1989; Luo et al., 1998). Because LBWT piglets are small, their absolute growth rate is not necessarily improved, but fractional growth, relative to initial body weight, is accelerated during the first month of life (Morise et al., 2009; Myrie et al., 2011; K. R. Poore & Fowden, 2004; Ritacco et al., 1997). Unfortunately, compensatory gain is often characterized by greater fat than lean tissue accretion (Barker et al., 2005; Dulloo et al., 2006; Gondret et al., 2006; K. R. Poore & Fowden, 2004). The relatively fatty pattern of compensatory gain is easily observable in pigs, where LBWT animals had smaller muscles and a higher percentage of body fat at slaughter (Gondret et al., 2006). The same experiment also showed that LBWT pigs grew more slowly and with reduced feed efficiency. Although compensatory gain is efficient early on, LBWT pigs gain weight less efficiently than NBWT pigs as they age because fat stores energy more densely, yet weighs less than muscle (Ji et al., 2017; Krueger et

al., 2014). The undesirable body composition resulting from compensatory gain increases risk of metabolic disease, creating debate as to whether or not it is desirable (Dulloo et al., 2006).

The leading theory as to the mechanism of compensatory gain, called the neuroendocrine model, essentially states that IGF-I production is suppressed, and tissue sensitivity to IGF-I increases during nutrient restriction (Griffin, 2015). Once nutrition is restored, IGF-I production resumes, and muscle and adipose tissue experience a period of hyper-anabolism (Griffin, 2015). Temporal patterns of circulating and local IGF-I in LBWT neonates support this hypothesis. Newborn LBWT infants had lower umbilical IGF-I (M. H. Lee et al., 2010), and LBWT neonatal pigs had lower circulating IGF-I than NBWT pigs (Davis et al., 1997; Schoknecht et al., 1997). Neonatal LBWT pigs also had decreased IGF1 mRNA expression in skeletal muscle (Y. Chen et al., 2016). However, LBWT neonatal pigs are born with increased expression of IGF-I receptors in skeletal muscle (Y. Chen et al., 2016). Although LBWT pigs are born with low IGF-I, it quickly normalizes by 2 days of age (Ritacco et al., 1997). Increasing IGF-I has the potential to stimulate growth. When neonatal LBWT pigs received a constant infusion of IGF-I for 7 days, protein, fat and body weight gain were restored to normal levels (Schoknecht et al., 1997). In fact, growth rate in LBWT infants is strongly correlated with circulating IGF-I concentration (Kajantie et al., 2002; Thieriot-Prevost et al., 1988).

LBWT infants are usually provided high energy, high protein diets to support compensatory gain (Thureen & Heird, 2005). However, the benefits of extra nutrients remain debatable. Protein and energy enriched diets have resulted in some moderate growth improvement of LBWT human infants, but outcomes have been inconclusive (Kashyap et al., 1988; Young et al., 2012). In neonatal pigs, protein and energy rich diets improved muscle

growth in both LBWT and NBWT pigs compared to adequately nutritious diets (Hu et al., 2018). Older LBWT pigs, in the grower phase, did not experience compensatory gain in response to nutrient dense diets (Douglas et al., 2014). To support compensatory gain, estimated protein intake requirements for LBWT infants are very high (Embleton et al., 2015). Increased dietary protein may be effective without supplemental energy, although its inclusion must be limited to avoid acidosis and uremia (Fenton et al., 2014). Formulas with increased protein, with other nutrients constant, increased growth and protein accretion in LBWT infants compared to lower protein formulas (Fenton et al., 2020). However, the positive effect of high protein formula on growth rate may not be specific to LBWT animals. High protein formula increased growth rate of both LBWT and NBWT piglets (Morise et al., 2011). The BCAA are also being specifically investigated because of low circulating concentrations in neonatal blood (He et al., 2011; Sanz-Cortés et al., 2013). If BCAA alone could cause the same growth increase as high protein diets, the risk of acidosis and uremia could be reduced. To date, there have been few studies in which BCAA are supplemented to LBWT neonates long enough to observe changes in growth rate. Leucine increased growth rate of weaned LBWT pigs, with no effect on NBWT pigs (Xu et al., 2016). However, the effect of leucine may have been confounded by increased feed intake. In another experiment, BCAA supplementation had no effect on growth of neonatal pigs (Manjarín et al., 2018).

Diets high in energy, with only adequate protein, may also increase growth, but result in greater fat gain than high protein diets (Kashyap et al., 1988). Considering the lasting metabolic disadvantages associated with LBWT, overfeeding can be detrimental in the long run. In fact, nutrient restriction to LBWT rat pups improved metabolic outcomes including insulin sensitivity

and hepatic lipid metabolism (Dai et al., 2012; Garg et al., 2013). Energy is supplied milk replacement formulas for LBWT infants as lactose or other simple carbohydrates, and lipid emulsions, which are traditionally a mix of palm, soybean, canola, and fish oils to approximately mimic the fatty acid composition of breast milk (Zou et al., 2016). Coconut oil and medium-chain triglycerides are increasingly included up to 30-40% of fat to replace vegetable oils because they contain fewer phytosterols than soybean oil, and are more digestible than the long-chain fatty acids in traditional oils (W. W. Hay & Hendrickson, 2017).

LBWT infants are more likely to have "feeding intolerance" because of their underdeveloped gastrointestinal tracts. Infants with feeding intolerance can receive nutrients injected directly into their blood stream, called parenteral nutrition (Fanaro, 2013). Although parenteral feeding is a necessary and effective solution for feeding intolerance, bypassing the gastrointestinal tract creates new problems. Lipids are normally transported through vasculature in soluble lipoprotein particles secreted from the small intestine or liver, which have surface proteins that interact with tissues and facilitate their uptake. Emulsified lipid particles mimic lipoproteins, but lack membrane proteins and consequently accumulate in the liver (Nandivada et al., 2013). Therefore, parenteral feeding often results in cholestasis, fat accumulation, and cirrhosis in the liver (Madnawat et al., 2020).

Fats

Overview

Lipids are water insoluble organic molecules. Nutritionally important lipids include fatty acids, cholesterol, and fat-soluble vitamins. Fatty acids are hydrocarbon chains with one carboxyl group. Fatty acids in plants and animals are mostly found esterified to glycerol. Three

fatty acids attached to a glycerol backbone form a triacylglycerol (TAG), which function to densely store fatty acids for future energy production. Two fatty acids esterified to glycerol, with a phosphate-linked, polar head group, form a phospholipid. Phospholipids are the basic components of cell membranes. Fatty acids are an excellent source of energy, although they are not technically essential nutrients, with the exception of the omega-6 polyunsaturated fatty acid linoleic acid. Linoleic acid is essential for its roles in inflammation and membrane composition, rather than energy provision.

Fatty acids vary in chain length and degree of saturation, both effecting their function. Chain length is also used to classify fatty acids. Under this classification fatty acids of 2 to 6 carbons are classified as short-chain fatty acids, or volatile fatty acids. Short-chain fatty acids provide usable energy and are primarily synthesized by gut bacteria and can be absorbed and used by the host. Medium-chain fatty acids (MCFA) are from 8 to 14 carbons long. Animals do not synthesize very many MCFA, with exception of the mammary gland producing them for milk production. MCFA are more soluble than longer fatty acids, and are more digestible (Odle, 1997). Fatty acids of 16 carbons and longer are long-chain fatty acids (LCFA). The majority of fatty acids in animal and most plant tissue are LCFA.

Saturation of fatty acids refers to the maximum number of hydrogen molecules being covalently bound to the hydrocarbon chain. Each carbon in a chain is bound to two other carbons. When those bonds are single bonds, two unpaired electrons are left on each carbon to bind with hydrogen atoms. When there is a double bond between carbons, only one hydrogen may bind with each carbon sharing the bond, and the fatty acid is termed “unsaturated”. Double bonds create flexible kinks in the hydrocarbon chain which effect its physical properties. Animals only express the enzymes to insert a double bond 9 carbons in from the methyl, or

“omega” end of a saturated fatty acid (Nagao et al., 2019). The resulting fatty acid would be an “omega-9” monounsaturated fatty acid. Consequently, omega-3 and omega-6 polyunsaturated fatty acids must be consumed in the diet, although omega-3 polyunsaturated fatty acids are not officially designated essential.

Each fatty acid is named according to its length and saturation. There is also a common abbreviation system for this information. For example, oleic acid is an 18 carbon-long monounsaturated fatty acid, abbreviated C18:1. The “C” stands for carbon, followed by number of carbons in the molecule, then a colon and the number of double bonds.

Digestion, Catabolism, and Synthesis

The digestion of fatty acids is relatively complex because they are insoluble in the aqueous environments of the intestinal lumen, cytosol, and blood. In the stomach and small intestine, lipases hydrolyze fatty acids from TAG and phospholipids, generating free fatty acids, monoacylglyerols, and lysophospholipids (Whitcomb & Lowe, 2007). The polar ends of fatty acids, lysophospholipids, monoacylglycerols and cholesterol, together with bile acids secreted from the gallbladder, interact with the aqueous environment, and form a membrane surrounding their hydrophobic ends and other lipids (E. Bauer et al., 2005). These particles are called mixed micelles and are able to diffuse across enterocyte membranes without transport. Inside enterocytes, fatty acids are transported through the cytosol by fatty acid binding proteins (FABP) (Glatz & Veerkamp, 1985). Enterocytes express FABP2, and hepatocytes express FABP1 (Mansbach & Siddiqi, 2010). Free fatty acids are reesterified to mono/diacylglycerols and lysophospholipids to reform TAG and phospholipids. TAG, phospholipids, and other lipids are compiled into lipoprotein particles, particularly chylomicrons. Chylomicrons are very low-

density particles comprised of dietary lipids, with a phospholipid and cholesterol monolayer surrounding a TAG and cholesterol ester interior (Mansbach & Siddiqi, 2010). Chylomicrons function to solubilize lipids for transportation from the small intestine via the circulatory system. Chylomicrons are secreted into the lymph, where they reach general circulation at the thoracic duct. The protein lipoprotein lipase, expressed on the capillary epithelium of muscle and adipose tissues, hydrolyzes fatty acids from chylomicrons and very low density lipoprotein (VLDL) (H. Wang & Eckel, 2009). Fatty acids hydrolyzed from by lipoprotein lipase, as well as circulating non-esterified acids bound to albumin, primarily enter cells facilitated by transport proteins, the best characterized of which is fatty acid translocase (CD36) (I. J. Goldberg et al., 2009). Fatty acids are utilized according to the cell's needs. Excess fatty acids are reesterified to TAG and phospholipids which are incorporated into tissues. Chylomicron remnants return to the liver.

Fatty acids are hydrolyzed from TAG when energy production is required during fasting. Free fatty acids from adipose tissue enter circulation bound to albumin to become available to active tissues like skeletal muscle (Rosen & Spiegelman, 2006). The first step towards oxidizing a fatty acid for energy production is activation by coenzyme A. Fatty acyl-CoA are transported across the two mitochondrial membranes via the carnitine cycle, which is facilitated and regulated by carnitine palmitoyltransferase 1 (CPT1) on the outer mitochondrial membrane (Schulz, 1991). In the mitochondrial matrix, acyl-CoA of various lengths are broken down to two-carbon acetyl-CoA units. This process, called β-oxidation, is catalyzed by a series of four enzymes, and reduces one FAD and one NAD+ for each acetyl-CoA produced. $FADH_2$ and NADH produce ATP via the electron transport chain, and acetyl-CoA is oxidized to CO_2 and generates further reducing equivalents via the TCA cycle (Schulz, 1991). Complete oxidation of one 16 carbon palmitate molecule generates 129 ATP molecules, far more than one molecule of

glucose or any amino acid. It is important to note that acetyl-CoA is a common metabolite of fatty acids, glucose, and many other substrates.

Animals synthesize fatty acids so that excess carbon from glucose and lipogenic amino acids can be stored efficiently. The liver and adipose tissue are capable of lipogenesis, but the primary location differs between species. Humans and rats primarily synthesize fatty acids de novo in the liver, whereas pigs synthesize most fatty acids in adipose tissue (Laliotis et al., 2010; Vernon et al., 1999). De-novo lipogenesis occurs in the cytosol, where the enzyme acetyl-CoA carboxylase elongates an acetyl-CoA into the 3-carbon malonyl-CoA (Sanders & Griffin, 2016). The ATP dependent acetyl-CoA carboxylase reaction commits the carbon substrate to lipogenesis and is highly regulated. The product malonyl-CoA is a potent inhibitor of CPT1, thereby inhibiting β-oxidation (Saggerson, 2008). Malonyl-CoA is the substrate for the fatty acid synthase enzyme complex. Through a series of reactions, fatty acid synthase starts with one acetyl-CoA and elongates it two carbons at a time with malonyl-CoA, releasing CO_2 and oxidizing one NADPH, until the new fatty acid is 16 carbons in length (palmitate), with the exception of mammary cells which allows early termination to generate a range of fatty acids. In other tissues, most fatty acids synthesized by animals begin as palmitate, and from there can be shortened, elongated, and desaturated by other enzymes.

Metabolism of Medium-Chain Fatty Acids

The physical qualities of MCFA create stark differences in the way they are digested compared to LCFA. Firstly, medium-chain TAG are more digestible than long-chain TAG because they are hydrolyzed more quickly in the lumen and can also diffuse into enterocytes intact (Odle, 1997). In enterocytes, MCFA are unlikely to be reesterified to TAG and

phospholipids, and therefore are not packaged in chylomicrons. Instead, MCFA travel directly to the liver via the portal vein bound to albumin (Marten et al., 2006). Similar to their metabolism in enterocytes, MCFA are not well reesterified in hepatocytes or exported to circulation in VLDL. Rather than being stored as TAG, MCFA can diffuse into mitochondria, without requiring CPT-1 transport, thereby bypassing a major regulatory step in β-oxidation (Rasmussen et al., 2002). Therefore, β-oxidation is considered the only fate of MCFA, a phenomenon termed "obligatory oxidation". The theory of obligatory oxidation was proposed when rats fed a high fat diet gained less weight when a portion of fat was replaced with medium-chain TAG (Baba et al., 1982; Geliebter et al., 1983). Although there is abundant evidence that MCFA are more digestible and metabolizable than LCFA (Odle, 1997), oxidation of MCFA is never actually 100% in vivo (Sulkers et al., 1989). Furthermore, oxidation of MCFA is chain-length dependent between fatty acids 7 to 10 carbons in length (Odle, 1997), and the principles of obligatory oxidation which apply to shorter MCFA may not apply to the most abundant fatty acid found in coconut oil, lauric acid (C12:0).

Although there is evidence of obligatory oxidation of shorter MCFA, lauric acid may be an exception. In isolated rat hepatocytes incubated with 0.1 mM lauric acid, the majority of lauric acid is oxidized to acid soluble metabolites by 4 hours, but only about 10% is fully oxidized to CO_2 (Christensen et al., 1991). However, up to 25% of lauric acid was stored in TAG (Christensen et al., 1991). A similar experiment found 39% of lauric acid was oxidized to acid soluble intermediates and CO_2 in rat hepatocytes, and 25% was stored in lipids, with 10% as TAG (Rioux et al., 2003). Interestingly, the vast majority of lauric acid retained in hepatocytes was elongated to myristic (C14:0), palmitic, and stearic acid (C18:0) by 4 hours of incubation (Rioux et al., 2003).

Metabolic Disease

Branched-Chain Amino Acids and Metabolic Dysfunction

Obesity is a major risk factor for many diseases, including type 2 diabetes, coronary heart disease and metabolic syndrome (Nguyen et al., 2008). Metabolic syndrome is a condition which includes central obesity, insulin resistance, atherogenic dyslipidemia, high blood pressure, general inflammation, and a prothrombotic state (Grundy et al., 2004). Obesity and insulin resistance are both associated with increased circulating free fatty acids and ectopic fat deposition. Obesity increases circulating free fatty acids because adipocytes, which safely store fatty acids under normal conditions, are overloaded (Coppack et al., 1992). Other tissues including skeletal muscle and the liver end up storing fat improperly, which contributes to insulin resistance (Lettner & Roden, 2008). Insulin resistance, incidentally, increases free fatty acid production from adipose tissue independently of obesity or overnutrition, as a mechanism to provide energy to tissues which are unable to adequately detect glucose availability. Therefore, insulin resistance also increases risk of ectopic fat deposition and development of nonalcoholic fatty liver disease (NAFLD) (Gaggini et al., 2013). Obesity, insulin resistance, and NAFLD, but not necessarily metabolic syndrome, are the metabolic dysfunctions associated with plasma BCAA concentration.

Ever since plasma BCAA concentrations were discovered to be elevated in obese people (Felig, 1970), researchers have investigated whether BCAA cause obesity and metabolic dysfunction. The relationship between BCAA and obesity is complex. BCAA have some metabolic benefits in healthy animals including prevention of adiposity (Freudenberg et al., 2012; H. Li et al., 2012a; Vianna et al., 2012) and improved glucose homeostasis (Binder et al.,

2013; Eller et al., 2013; Zhang et al., 2007). However, in obese or high fat-consuming animals, BCAA can exacerbate issues with glucose clearance (Newgard et al., 2009; Sunny et al., 2015). One of the most influential studies linking BCAA to obesity was by Newgard et al. (2009). In that study, a metabolomics analysis of plasma taken from obese individuals indicated an elevated plasma BCAA concentration compared to nonobese individuals. To explore the positive correlation between obesity and plasma BCAA, rats were fed one of 4 diets either high fat or standard chow, supplemented or un-supplemented with BCAA. Rats fed high fat, BCAA supplemented diets had lower glucose tolerance than the other treatments, but BCAA did not affect glucose tolerance in the standard chow diet and never increased body weight gain (Newgard et al., 2009). Furthermore, the effect of BCAA on insulin resistance may not translate to other species. The same researchers found that BCAA had no additional effects on insulin resistance in mice fed high fat, high fructose diets (J. Lee et al., 2021a). The authors proposed that the mice did not develop the same phenotype as rats fed high fat and BCAA diets because of species specific differences in fat metabolism. The effect of BCAA on insulin resistance may be mTOR dependent. Rapamycin improved glucose tolerance to normal in high fat, BCAA fed rats in Newgard et al. (2009). In cultured muscle cells, an amino acid mixture decreased glucose uptake by decreasing IRS-1 associated P13K activity, which was reversed when incubated with rapamycin (Tremblay & Marette, 2001). Based on available research, BCAA may contribute to insulin resistance, which may account for their correlation with obesity.

Conversely, obesity and insulin resistance may affect BCAA catabolism. For example, BCAA concentrations are elevated in plasma of genetically obese and insulin resistant rodents without dietary supplementation of BCAA (Maida et al., 2017; She et al., 2007; Zhou et al., 2019), and high fat, high sugar diets increased BCAA concentration in porcine plasma (Polakof

et al., 2018) and livers (Frano et al., 2019). High fructose diets caused insulin resistance without obesity in mice, and decreased transaminase activity in skeletal muscle and increased plasma BCAA (David et al., 2019). Furthermore, BCAA catabolizing enzymes increased, and circulating BCAA decreased following weight loss in human adults who underwent gastric bypass surgery (Laferrère et al., 2011; Magkos et al., 2013). These examples suggest that insulin resistance is the common cause of increased plasma BCAA. Figure 1.5 summarizes the proposed mechanisms by which insulin resistance decreases catabolism of BCAA. Altogether, BCAA and insulin resistance appear to have a circular relationship.

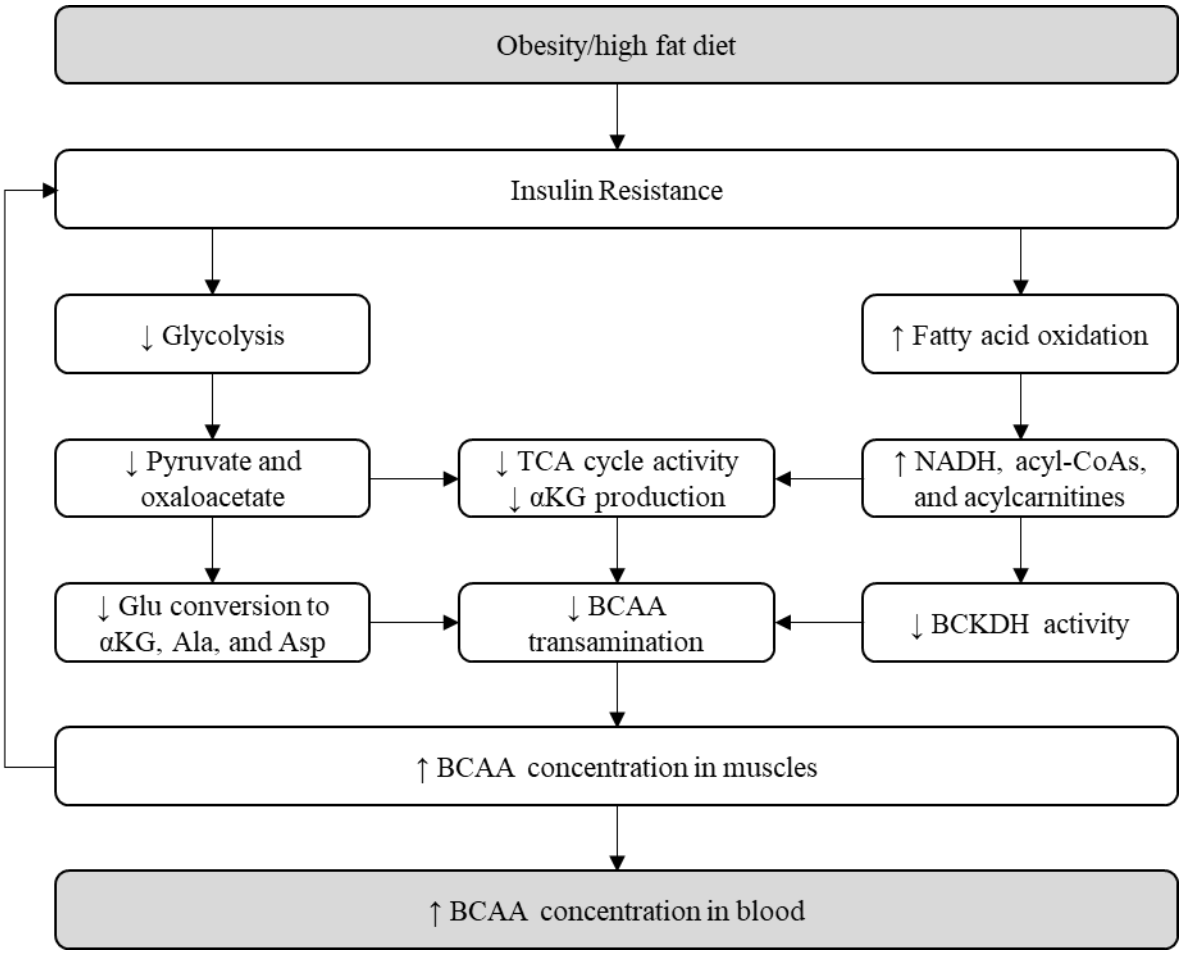


Figure 1.5. Proposed mechanisms of insulin resistance causing increased circulating BCAA concentrations. Glu, glutamate; αKG, α-ketoglutarate; BCAA, branched-chain amino acid, BCKDH, branched-chain keto acid dehydrogenase.

Pediatric Nonalcoholic Fatty Liver Disease

If BCAA are supplemented to infant formulas to improve neonatal growth, it is important to understand whether it could contribute to neonatal metabolic disorders. Pediatric nonalcoholic fatty liver disease (NAFLD) affects 10% of school-aged children, and 44-77% of obese children in western countries (Fitzpatrick, 2019). Factors contributing to pediatric NAFLD include genetic predisposition, nutrition, insulin resistance, oxidative stress, bile acid metabolism, and gut microbiota (Fitzpatrick, 2019). Risk of pediatric NAFLD begins in the neonatal period. Fetal livers can be predisposed to NAFLD by maternal obesity and energy transfer (Baker & Friedman, 2018). During the neonatal period, energy metabolism adjusts from umbilical glucose to oxidizing fatty acids from milk (Girard et al., 1992). To accommodate increased fat metabolism, mitochondrial content in the liver increases in neonatal period (Baker & Friedman, 2018).

Considering the prevalence of and need for improved therapies for NAFLD, it is an important area of research for human health. Generating controlled experimental data on NAFLD is challenging due to the invasiveness of acquiring liver samples. The variety of causes and the gradual, progressive nature of NAFLD onset make it especially difficult to recreate the human mechanisms of NAFLD in animals. There is no single standard animal model of NAFLD (Jahn et al., 2019). In animal models, fatty liver can be induced either by genetic modification or by diet. Genetic models include leptin or leptin receptor deficient mice, which develop simple steatosis secondary to obesity, and low-density lipoprotein receptor (Bieghs et al., 2012) or apolipoprotein E-deficient mice (Schierwagen et al., 2015), which become hypercholesterolemic

and are prone to nonalcoholic steatohepatitis (Ioannou, 2016). Dietary methods of inducing NAFLD include high fat or fructose diets, and low folate, methionine, or choline deficient diets. High fat obesogenic diets are relatively slow to cause NAFLD, but best mimic metabolic and histologic features of the human condition with obesity, insulin resistance, liver inflammation and fibrosis. Cholesterol and fructose help ignite inflammation in high fat diets (Radhakrishnan & Pellizzon, 2020). Diets deficient in both methionine and choline induce steatosis more rapidly, but work by impairing secretion of VLDL (Kulinski et al., 2004; Leclercq et al., 2000; Weltman et al., 1996; Yao & Vance, 1988). In contrast to NAFLD associated with metabolic syndrome, methionine and choline deficient diets actually cause increased insulin sensitivity and weight loss (Rinella & Green, 2004; Rizki et al., 2006). Existing models are therefore limited in what they can tell us about disease development and progression but may still help to understand the effects of fat accumulation on liver function.

Neonatal pigs are the preferred animal model in infant nutritional research due to their metabolic similarities and large size which allows catheterization. However, models of NAFLD in pigs are less developed than those in mice. Existing models in rodents and pigs take too long to develop, so that the animals are no longer pediatric. Pigs fed a high fat diet from 4 to 7 months of aged did not develop NAFLD, suggesting young pigs are more resistant than humans to liver lipid accumulation in response to high fat diets (Sejersen et al., 2013). When fructose was added to high fat diets of neonatal, leptin resistant Iberian pigs developed NAFLD after 10 weeks (Smith & Melnyk, 2019). Although diets high in fat and fructose did not induce NAFLD in 5-10 month old Ossabaw mini pigs after 24 weeks of feeding, NAFLD was induced by a diet high in protein, low in choline, and high in fat composed of hydrogenated soybean oil, coconut oil and lard (L. Lee et al., 2009). When 13 day old Ossabaw pigs consumed diets high in

fructose, cholesterol, and fat including coconut oil, they developed steatosis and NASH by 10 weeks (Hernandez et al., 2020). All of these models take at least 10 weeks to develop a NAFLD phenotype, which is too long for the study of neonatal pigs. Based on preliminary data from our laboratory, high fat diets sourced from coconut oil cause up to 25% liver fat accumulation in just 3 weeks. This model was used to study the effect of NAFLD on BCAA metabolism in the second experiment of this dissertation.

Chapter 2. Branched-Chain Amino Acid Supplementation Does Not Enhance Lean Tissue Accretion in Low Birth Weight Neonatal Pigs, Despite Lower Sestrin2 Expression in Skeletal Muscle

Abstract

Postnatal muscle growth is impaired in low birth weight (LBWT) neonatal pigs. Dietary leucine supplementation has been established as a dietary intervention to enhance muscle growth in growing animals. The aim of this study was to investigate the efficacy of supplementing LBWT neonatal pig diets with branched-chain amino acids (BCAA) to enhance the rate of protein accretion. Twenty-four pigs began the trial at 3 d of age. LBWT and normal birth weight (NBWT) were defined when birth weight was 2 SD below or within 0.5 of the litter average. Each LBWT pig was matched with a NBWT same sex littermate and assigned to either the Control diet or supplemented with 1% BCAA in a 2×2 factorial arrangement. Diets were isocaloric, isonitrogenous and met NRC requirements. Pigs were fed 250 ml/kg BW/day of Control or BCAA sow milk replacers. Carotid and jugular catheters were surgically placed under general anesthesia on day 21 of feeding for blood sampling. Body composition was measured by dual x-ray absorptiometry. On day 29, blood samples were taken over 4 h following a meal. Data were analyzed using MIXED procedure of SAS version 9.4. Plasma leucine, isoleucine, and valine concentrations were greater in pigs fed BCAA ($P<0.05$). LBWT pigs had lower weight gain ($P<0.001$) and average daily gain ($P<0.01$), but improved fractional rate of gain ($P<0.0001$), feed efficiency ($P<0.0001$) and efficiently of protein accretion ($P<0.0001$) than NBWT pigs regardless of diet. LBWT pigs had decreased expression of Sestrin2 ($P<0.01$) in the *longissimus dorsi*, but no corresponding increase in translation initiation signaling compared to NBWT pigs.

Although LBWT pigs had lower expression of *BCAT2* in the LD compared to NBWT pigs, which was increased to normal by BCAA supplementation ($P<0.05$), and increased hepatic expression of hepatic *BCKDHA* ($P<0.05$), plasma branched-chain keto-acid (BCKA) concentrations were only affected by diet ($P<0.0001$). Supplementary BCAA did not improve lean gain, and were catabolized. These data also show that LBWT pigs grow leaner and more efficiently than NBWT pigs when limit-fed.

Introduction

Intrauterine growth restriction is caused by a myriad of maternal and placental factors (Resnik, 2002), and is a serious issue in pediatric medicine (Mcmillen & Robinson, 2005; Saleem et al., 2011) and livestock production (Quiniou et al., 2002; Wu et al., 2006). Fetuses afflicted with intrauterine growth restriction (Damodaram et al., 2012; Kilavuz & Vetter, 1999) are not only born weighing less than what is considered normal for their gestational age but also suffer from a reduction in neonatal growth rates, and are thought to be at greater risks of morbidity and mortality in infancy and metabolic disorders later in life (Barker, 2000; Godfrey & Barker, 2007; Mcmillen & Robinson, 2005). Although low-birthweight (LBWT) neonates could achieve compensatory growth during childhood (Soto et al., 2003), it is not yet clear why they fail to fully "catch-up" to their normal birth weight (NBWT) counterparts.

It is well established that muscle growth occurs by hyperplasia and hypertrophy and that hyperplasia is completed in utero, and as a result skeletal muscle fiber numbers are fixed by birth (Glass, 2003; Miyazaki & Esser, 2009). The reduction in fiber numbers reported in LBWT neonates compared with their NBWT counterparts (Greenwood et al., 2000; Nissen et al., 2003) may limit lean mass accretion during compensatory growth (Handel & Stickland, 1988). Data gleaned from animal models also suggest that LBWT is associated with greater expression of oxidative muscle fibers as compared with greater expression of glycolytic fibers in NBWT neonates (Handel & Stickland, 1988), which is believed to cause greater protein turnover rates, and result in a reduction in growth efficiency (Denne et al., 1991; Garlick et al., 1989).

Muscle fiber hypertrophy occurs by two mechanisms. The first mechanism is through myonuclear accretion mediated by satellite cell fusion with existing myofibers (Wozniak et al.,

2005). In this regard, LBWT pigs have fewer satellite per gram of skeletal muscle than their NBWT littermates at 4 days of age (Stange et al., 2020), and thus have a diminished ability to increase their myonuclear domain. In addition, we have previously reported that satellite cells isolated from skeletal muscle of LBWT pigs have a diminished ability to fuse compared with those of NBWT siblings (Y. Chen, Zhu, et al., 2017). The second mechanism is through regulation of protein synthesis and degradation rates, a process in which protein accretion occurs when protein synthesis exceeds degradation. The rate limiting step in protein synthesis is translation initiation regulated by mechanistic target of rapamycin (mTOR) (Ma & Blenis, 2009) through insulin and leucine dependent mechanisms (Anthony, Anthony, et al., 2000; Suryawan et al., 2008).

We are using the neonatal pig as a model for the human infant due the natural prevalence of LBWT and NBWT pigs in the same litter which reduces genetic and parental influence. We have also reported that downregulated translation initiation signaling in skeletal muscles at birth predisposes LBWT pigs to slow lean tissue accretion (Y. Chen, McCauley, et al., 2017; El-Kadi, Chen, et al., 2018). It is not known whether changes in translation initiation signaling persist long-term, and whether leucine supplementation would ameliorate skeletal muscle protein deposition in LBWT pigs via an upregulation of mTOR signaling pathway. We hypothesized that branched-chain amino acid (BCAA) supplementation would improve lean tissue accretion in neonatal pigs by upregulating translation initiation. Thus, the objectives of the current study were to: 1) investigate the effect of BCAA supplementation on growth of LBWT and NBWT pigs, 2) determine changes in the regulation of translation initiation in response to BCAA supplementation, and 3) identify whether BCAA supplementation influence BCAA degradation pathways.

Materials and Methods

Animals. All experimental procedures were approved by Virginia Tech Institutional Animal Care and Use Committee. Twenty-four cross-bred, sex-matched littermates were selected from 7 litters based on weight at birth, where pigs weighing ≤ 2 SD or within 0.5 SD from the litter mean were considered LBWT and NBWT (Y. Chen, McCauley, et al., 2017; El-Kadi et al., 2019). Pigs were allowed to suckle colostrum for 24 hours before being moved to individual cages kept in rooms maintained at 30°C with additional heat lamps.

Diets. A sow milk replacer (Table 2.1) basal mix was formulated (National Research Council, 2012) and supplemented with 1% BCAA (BCAA diet; 1:0.68:0.5 leucine:isoleucine:valine) or an equimolar amount of nitrogen from alanine (Control diet; 0.7% alanine). All pigs were fed the Control formula during a 2-day adaptation period before being assigned to one of the two dietary groups in a 2×2 factorial arrangement of diet and birthweight. Pigs were fed the experimental diets at 250 mL· kg body weight -1 · d-1 in 5 equal meals every 4 hours from 8:00am to 12:00am. Pigs were weighed every other day and feed allotment adjusted accordingly.

Surgeries and Blood Sampling. On day 21 of feeding, pigs were surgically fitted with indwelling jugular and carotid catheters under general anesthesia by isoflurane (El-Kadi et al., 2019). On the last day of feeding (day 28), blood samples were taken in heparinized syringes from the arterial catheter at 0, 15, 30, 45, 60, 120, 180, and 240 minutes following the first meal of the day. Pigs received a second meal and were euthanized 60 minutes later.

Amino and keto acid analysis. Plasma was immediately separated from blood by centrifugation (3200 × g for 10 min). Amino acid concentrations in plasma and longissimus muscle were determined gravimetrically using an internal standard containing [U-13C, 15N]

amino acids and [15N2] urea (Cambridge Isotope Laboratories, Inc, Andover, MA). Amino acids were converted to their t-butyldimethylsilyl derivatives (Calder et al., 1999; El-Kadi et al., 2006) and branched-chain α-keto acids to quinoxanol t-butylmethylsilyl derivatives (Calder & Smith, 1988) and quantified by chromatography-mass spectrometry (Agilent 5975 Mass Selective Detector; Agilent Technologies, Wilmington, DE) fitted with a HP-5MS fused silica capillary column (Agilent Technologies).

Body composition by Dual-energy X-ray absorptiometry. Body composition was determined one day prior to initiation and termination of feeding using Dual-energy X-ray absorptiometry (DXA) whole body scanner (Lunar Prodigy; GE Medical systems, Milwaukee, WI). Pigs were fasted overnight and placed under light isoflurane sedation for the duration of the scan. Percent lean, fat, and bone minerals on day 28 were calculated from the product of percentage lean, fat or bone mineral at the time of the scan adjusted for body weight on day 28 as previously described (El-Kadi, Boutry, et al., 2018). Protein deposition was calculated using the equation (Mitchell et al., 1998):

$$\text{Body protein} = -1.062 + (0.2 \times \text{DXA lean})$$

Energy deposition was calculated using protein and fat deposition using 5,680 and 9,460 kcal • kg^{-1} as the caloric content of protein and fat (El-Kadi, Boutry, et al., 2018). Efficiency of energy and protein retention were calculated from energy and protein retained and total dietary energy and protein consumed over the duration of the study (El-Kadi, Boutry, et al., 2018).

Total RNA and protein content. Total RNA and protein were extracted from longissimus muscle samples homogenized in 5N perchloric acid. RNA concentration was quantified using a spectrophotometer (NanoDrop, Thermo Fisher Scientific, Wilmington, DE). Protein was

solubilized with NaOH, and quantified colorimetrically using a bicinchoninic protein assay kit (Thermo Scientific, Rockford, IL).

Quantitative real-time PCR. RNA was extracted from longissimus and liver using Direct-zol RNA Miniprep kit (ZYMO Research, Orange, CA). First strand cDNA was generated from RNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Samples were mixed with primers (Table 2.2) and Fast SYBR Green (Applied Biosystems, Foster City, CA) and run in triplicate on 96-well plates using an ABI 7500 Fast Real Time PCR system (Applied Biosystems, Foster City, CA). All primers were validated by agarose gel electrophoresis, amplification efficiency curve, and product sequencing. Suitability of housekeeping genes was assessed using the Normfinder add-in for Microsoft Excel (Anderson et al., 2004). mRNA abundance relative to the NBWT Control group was quantified using the 2-ΔΔCt method relative to the geometric mean of H3F3A and PPIA in the longissimus and of TBP and B2M in the liver.

Protein abundance and phosphorylation. Abundance and phosphorylation of signaling proteins was performed by SDS-PAGE followed by immunoblotting using specific antibodies (Supplemental Table 2.1) as previously described (Y. Chen, McCauley, et al., 2017). Abundance of α-Tublin was used as a loading control and the phosphorylated proteins were normalized to their corresponding non-phosphorylated form.

Statistical analysis. Data were analyzed using SAS version 9.4 (SAS Inst. Inc., Cary, NC). All data were analyzed by ANOVA for a 2×2 factorial using the PROC MIXED procedure, with litter and sex as random effects. Temporal effects were determined using repeated measures analyses. Various autocorrelation structures were tested based on best fit using Bayesian Information Criterion. First-order Autoregressive gave the best fit and was used

for all subsequent analyses. When a significant effect was detected, means were compared using the Tukey-Kramer post hoc test. Data are expressed as least square means ± SEM and differences considered significance at $P \leq 0.05$.

Results

Body weight and growth performance. Body weight increased throughout the feeding period and was greater for NBWT pigs compared to their LBWT counterparts ($P < 0.001$; Figure 2.1A), but was not improved by BCAA supplementation. However, fractional body weight calculated from the ratio of body weight at time t relative to weight at initiation of feeding, was greater for LBWT compared to NBWT pigs on days 24 to 28 indicating compensatory growth of LBWT group compared with their NBWT siblings ($P < 0.001$; Figure 2.1B). Although LBWT pigs gained (Table 2.3) on average less body weight than their NBWT siblings (4.65 kg compared with 5.65 kg; $P < 0.01$), LBWT pigs consumed less food than NBWT pigs (18.5 vs 25.5 L; $P < 0.001$), and this resulted in improved food conversion efficiency for LBWT compared with NBWT siblings (0.25 vs 0.22 kg • L^{-1}; $P < 0.001$).

Body composition. Fat mass, lean tissue mass and bone mineral content (Figure 2.2) determined by DXA were greater for NBWT than their LBWT siblings following 28 d of feeding ($P < 0.05$). Although fat mass as a percentage of body weight was greater for all pigs on day 28 than day 0 ($P < 0.001$), the percentage of fat was greater for NBWT than for LBWT pigs at days 0 and 28 ($P < 0.001$). Conversely, the percentage of lean mass was reduced at day 28 than at day 0, and remained greater for LBWT pigs compared with their NBWT siblings ($P < 0.001$). Bone mineral content as a percentage of body weight was less at day 28 than at day 0 ($P < 0.0001$), and

remained greater for NBWT than for LBWT pigs ($P < 0.01$). There was no effect of BCAA supplementation on body composition.

Efficiency of fat and protein retention. The efficiencies of fat and protein retentions were calculated from the ratio of fat and protein accretion determined by DEXA between the beginning and end of the feeding period over the amounts of energy and protein consumed from the diet over that period. Efficiency of energy retention (Table 2.4) was not affected by birthweight or diet and was 46.5 and 44% for LBWT and NBWT pigs. However, the efficiency of protein retention was greater for LBWT (75%) pigs compared with their NBWT (65.5%) siblings with no further improvement noted following BCAA supplementation.

Liver and muscle weights. Liver, longissimus dorsi, soleus and semitendinosus weights (Table 2.5) were greater for NBWT than LBWT pigs regardless of diet ($P < 0.01$). However, when liver and muscle weights were expressed as a percentage of body weight there was no effect of birthweight or BCAA supplementation.

Plasma amino acid concentrations. Plasma concentration of essential amino acids (Figure 2.3) peaked between 60 am 120 min post-feeding and returned to baseline by 240 min ($P < 0.05$). There was no effect of diet or birthweight on histidine or phenylalanine concentrations. As predicted, plasma isoleucine, leucine, and valine concentrations were greater for pigs fed the BCAA supplemented diet compared with those fed the control diet ($P < 0.05$), however, there was no effect of birthweight on the concentration of these amino acids. Plasma lysine concentration was greater for pigs fed the control than those fed the BCAA diet ($P < 0.05$). Concentration of methionine was greater for pigs fed the control compared with those fed the BCAA diet at 60 and 120 min ($P < 0.01$), and was greater for LBWT compared with their NBWT (91 vs 86 μmol • kg-1) siblings ($P < 0.05$). Threonine concentration was greater for pigs

fed the control compared with those fed the BCAA diet ($P < 0.001$). As predicted alanine concentration was greater (1170 and 496 μmol • kg^{-1}) for pigs fed the control compared with those fed the BCAA diet ($P < 0.0001$). Concentrations of all non-essential amino acids (Figure 2.4), except glycine, were greater following a meal and peaked between 45 and 120 min ($P < 0.05$). For those amino acids, concentrations were greater for pigs fed the control than those fed the BCAA diet ($P < 0.05$).

Muscle amino acid concentrations. Muscle free amino acid concentrations (Table 2.6) of isoleucine (0.66 and 0.41 μmol • g^{-1}), leucine (0.92 and 0.49 μmol • g^{-1}), and valine (1.15 and 0.59 μmol • g^{-1}) were as expected greater for pigs fed the BCAA supplemented compared with those fed the control diet ($P < 0.0001$). Conversely, alanine (2.30 vs 3.84 μmol • g^{-1}) was greater for pigs in the control group compared with their BCAA supplemented siblings ($P < 0.0001$). Concentrations of all other amino acids were not different between control and BCAA supplemented pigs or between LBWT and NBWT pigs.

Branched-chain amino acid catabolic gene expression and metabolites. Branched-chain amino acid transaminase 2 (BCAT2) mRNA expression (Figure 2.5) was reduced in the longissimus of LBWT pigs fed the BCAA diet compared with those fed the control diet, whereas for NBWT pigs fed either diet BCAT2 expression was intermediate ($P < 0.05$). No differences were noted for liver mRNA expression of BCAT2 among the groups. There was no difference in gene expression of alpha-keto acid dehydrogenase E1 subunit alpha (BCKDHA) in muscle, however, in the liver BCKDHA expression was greater for LBWT pigs compared with their NBWT siblings ($P < 0.05$). Plasma concentrations of α-ketoisocaproate (KIC), α-ketoisovalerate (KIV), and α-ketomethylvalerate (KMV) (Figure 2.6) were consistently greater for pigs fed the BCAA diet than those fed the control diet ($P < 0.0001$). However, while KIC and KMV

concentrations peaked by 120 min post-feeding, KIV concentration was reduced between feeding and 60 min after the meal before returning to pre-feeding concentrations. Despite the reduction in KIV immediately after feeding, the sum of all branched-chain keto acids peaked by 120 min, and was consistently greater for pigs fed the BCAA diet compared with those fed the control diet ($P < 0.0001$), however, there was no difference between LBWT and NBWT pigs.

Protein synthetic capacity. Total RNA content (Table 2.7) was greater for pigs fed the BCAA supplemented compared with those fed the control diet ($P < 0.05$). Protein content did not differ between LBWT and NBWT or between control and BCAA supplemented groups. However, protein synthetic capacity was greater for pigs fed the BCAA supplemented diet compared with those fed the control diet ($P < 0.05$), but did not differ between LBWT and NBWT pigs.

Intracellular signaling. In the longissimus dorsi muscle, protein expression and phosphorylation of AKT was not different among the groups (Figure 2.7). Although expression of Sestrin2 was greater for NBWT pigs compared with their NBWT siblings ($P < 0.01$), expression and phosphorylation of mTOR was not different among groups. Expression of 4EBP1 was greater for NBWT than for LBWT pigs ($P < 0.01$), however, 4EBP1 phosphorylation did not differ among groups. S6K1 expression and phosphorylation were not different among groups. Similarly, expression of Atrogin-1 and Murf1 were not different among groups (Supplemental Figure 2.2).

Discussion

It is well established that skeletal muscle protein deposition is greatest immediately after birth than at any other period in life (Davis et al., 1991, 1993, 1996; Reeds et al., 2000).

Although LBWT neonates undergo a period of compensatory growth during this period, they often fail to attain growth rates similar to their NBWT counterparts (Fitzhardinge & Inwood, 1989; Luo et al., 1998). We previously reported that downregulated translation initiation signaling in skeletal muscle at birth predisposes LBWT pigs to a reduction in lean tissue accretion (Y. Chen, McCauley, et al., 2017). In that study, it was unclear whether such changes in signaling observed at birth would remain long-term, and whether dietary interventions like BCAA supplementation would promote skeletal muscle growth. To eliminate the effect of feed intake on growth and to ensure a fast and complete consumption of formulas, pigs were limit fed the same amount of the two formulas adjusted for body weight.

In the current study, while fractional body weight of LBWT pigs was greater than their NBWT siblings from 24 to 28 days of feeding, their body weight remained less throughout the study. Compensatory growth occurs during the first year of life in LBWT infants (Beger et al., 2018; Soto et al., 2003), yet they do not always achieve similar height and body weight compared with their NBWT counterparts (Fitzhardinge & Inwood, 1989; Luo et al., 1998). In addition, LBWT infants would accrete more fat than their NBWT counterparts and this fat accretion is thought to contributes to development of metabolic disease later in life (Dulloo et al., 2010). Similarly, compensatory growth occurs in pigs (K. R. Poore & Fowden, 2004), and previous reports indicated that LBWT animals typically accreted fat at a disproportionately greater rate than their NBWT counterparts when fed ad libitum (Dulloo et al., 2006; Gondret et al., 2006; K. Poore & Fowden, 2002; K. R. Poore & Fowden, 2004). However, our current data suggested that lean tissue deposition improved while that of fat was reduced in LBWT compared with NBWT pigs indicating greater efficiency of protein deposition. The discordance among previous reports and the current results could be attributed to the amount of food offered. In the

current study pigs were limit fed to reduce the effect of feed intake on body composition compared with other studies in which food was offered ad libitum (Dulloo et al., 2006; Gondret et al., 2006; K. Poore & Fowden, 2002; K. R. Poore & Fowden, 2004). Our data are in support of previous reports that LBWT pigs limit-fed milk replacer gain weight more efficiently than NBWT pigs (Lecce, 1971). Furthermore, LBWT infants utilize dietary proteins efficiently and that feed efficiency in general is not negatively affected by birth weight during the neonatal period (Catzeflis et al., 1985; Kandil et al., 1991; Rigo & Senterre, 2006). Thus, the current data would suggest that while LBWT neonates are more efficient at depositing dietary protein compared with their NBWT counterparts, BCAA supplementation did not affect lean tissue deposition nor the efficiency of protein deposition.

A common feature of neonates afflicted with LBWT is asymmetric growth, in which growth of some organs like the brain is protected while that of muscles is greatly reduced (R. Bauer et al., 1998; Desai et al., 2005). We have previously shown that while skeletal muscle weight as a percent of body weight was less for LBWT pigs compared with their NBWT siblings at 14 d, liver weight was greater (El-Kadi et al., 2019). However, in the current study muscle and liver weights as a percent of body weight were similar indicating that these organs proportional to body weight after 4 weeks of feeding, and that BCAA supplementation had no effect of this growth. After 14 days, LBWT neonatal pigs no longer had a leaner body composition than NBWT pigs, and still had proportionally smaller LD (El-Kadi et al., 2019). High energy diets increased the percentage of LD weight in all pigs, demonstrating that dietary energy can limit muscle growth, but it was not limiting compensatory gain in LBWT pigs (El-Kadi et al., 2019). Similarly, LBWT pigs fed ad libitum had less skeletal muscle as a percentage of body weight than NBWT pigs by 28 days of age, and high protein diets had no effect (Morise

et al., 2011). Limit feeding in the current study may have prevented LBWT pigs from over-eating and accreting fat. However, ad libitum feed intake relative to body weight is not necessarily greater in LBWT production type piglets (Campbell & Dunkin, 1982; Morise et al., 2009), despite being slightly higher in newly weaned Yucatan miniature pigs (Myrie et al., 2011), and weaned commercial pigs (Krueger et al., 2014; Madsen & Bee, 2015). Furthermore, neither nutrient restriction (Hu et al., 2015), nor high nutrient intake (Han et al., 2013) interacted with the effect of birth weight on growth performance in neonatal pigs. More specific investigations into the nutrient requirements of LBWT pigs may be required to understand if nutrient requirements of LBWT animals are different from their NBWT counterparts.

The current paradigm is that leucine enhances protein accretion by improving protein synthesis and reducing protein degradation. Leucine enhances translation initiation by activating mTOR and translation initiation independently of insulin (Anthony, Anthony, et al., 2000; Crozier et al., 2005; Escobar et al., 2006). Leucine activates mTOR through a process thought to be mediated by binding to stress response element 2 (Sestrin2), an inhibitor of translation initiation. Sestrin2 binds with GTPase-activating protein activity towards Rags (GATOR) 2 to form a complex that suppresses GATOR1 and inhibits RAS-related GTP-binding protein (Rags) from activating mTORC1 (Suryawan & Davis, 2018; Wolfson & Sabatini, 2017).

Although the mechanisms driving compensatory growth are not fully understood, it is suggested that compensatory growth is strongly associated with IGF-I. For example, circulating IGF-I concentration is positively correlated with growth rate in very LBWT infants (Kajantie, 2003), and is it is a good indicator for predicting whether compensatory growth would occur in LBWT infants by 12 months (Thieriot-Prevost et al., 1988). Data gleaned from neonatal pigs suggest that at birth, LBWT pigs have reduced circulating IGF-I concentrations (Davis et al.,

1997; Schoknecht et al., 1997), and a concomitant reduction of IGF1 expression in skeletal muscle (Y. Chen, McCauley, et al., 2017). However, the reduction in blood and local muscle IGF-I expression is counteracted by a greater expression of IGF1R in skeletal muscle compared to NBWT pigs (Y. Chen, McCauley, et al., 2017; Tilley et al., 2007). Although the expression and phosphorylation of ERK1/2 was not affected by birthweight, S6K1 phosphorylation, and formation of the active eIF4E:eIF4G complex were reduced in muscles of LBWT pigs compared with their NBWT counterparts and seem to indicate that these changes may predispose LBWT pigs to a reduction in muscle hypertrophy (Y. Chen, McCauley, et al., 2017; El-Kadi, Chen, et al., 2018). While circulating IGF-I concentration returns to normal in LBWT pigs as early as 2 days of age and as such may suggest that compensatory growth in LBWT pigs is not mediated by IGF-I (Ritacco et al., 1997), it is still not clear whether local expression of IGF1 in muscle or IGF-IR expression would also return to normalcy during that timeframe.

It is well established that leucine acutely activates translation initiation and protein synthesis following a meal (Crozier et al., 2005; Escobar et al., 2006; Murgas Torrazza et al., 2010) independently of insulin (Anthony, Anthony, et al., 2000; Anthony, Yoshizawa, et al., 2000), but evidence in support of its therapeutic application for increasing lean accretion in infants is limited. Leucine has potential application for infants who require continuous tube feeding, as parenteral leucine pulses increased lean gain in continuously fed neonatal pigs (Boutry et al., 2016). However, there is less evidence of the effect of leucine on lean tissue accretion with oral supplementation. For example, suckling piglets given leucine supplements twice daily had greater body weight gain over those that are not supplemented over 14 days (Y. Sun et al., 2015). However, no further measurements were made to support whether weight gain was the result of muscle hypertrophy or body composition difference. In addition, it is possible

that leucine was limiting as a substrate for protein synthesis due to variable and insufficient supplies from sow milk (Rezaei et al., 2013). Others proposed that lighter weaned pigs have a higher requirement for leucine than their heavier counterparts (Bertocchi et al., 2019). In support of this concept, leucine increased feed intake, average daily gain, and translation initiation of weaned LBWT but not NBWT pigs (Xu et al., 2016). Obviously, there are several possible experimental differences that could explanations to explain the discrepancy between our results and those from previous reports and preclude direct comparisons. For example, we opted to feed pigs a constant amount of formula that contained a fixed proportion of BCAA whereas in other studies pigs were fed ad libitum and dosed with a fixed amount of leucine (Y. Sun et al., 2015). In addition, it is also possible that difference in age of the pigs would have precluded observing any differences since pigs in the current study were fed between birth and 4 weeks of age, whereas pigs were 3 weeks old and weaned to solid feed in other studies (Xu et al., 2016, p. 20). Regardless, our data are in support of other reports using neonatal pigs of similar age to those used in the current study suggest that BCAA supplementation has no effect on improving body weight of pigs (Columbus et al., 2015; Manjarín et al., 2016, 2018).

We previously reported that while there was no difference in mTOR phosphorylation between LBWT and NBWT pigs, phosphorylation of S6K1 was impaired in LBWT pigs due to decreased expression of eIF3e (Y. Chen, McCauley, et al., 2017), a subunit of a protein which serves as a scaffold for the phosphorylation of S6K1 by mTOR (Holtz et al., 2005). In the current study eIF3e protein expression (Supplemental figure 2.5) and S6K1 phosphorylation were not different between LBWT and NBWT pigs, suggesting eIF3 expression no longer limits translation initiation by 32 days of age. The current data also indicated that supplementation with BCAA had no effect on translation initiation signaling or lean tissue accretion despite

increasing intramuscular BCAA concentration and protein synthetic capacity. While the lack of difference between LBWT and NBWT pigs may be explained by the lack of differences in translation initiation components downstream of mTOR, these results were still unexpected for two reasons. The first is that while BCAA supplementation enhanced S6K1 and 4EBP1 phosphorylation and the formation of the active eIF4E:eIF4G complex formation albeit without affecting fractional rate of protein synthesis (Manjarín et al., 2016, 2018), in the current study BCAA supplementation did not enhance translation initiation signaling. The second, is that Sestrin2 a negative regulator of translation initiation signaling (Suryawan & Davis, 2018) was expressed less in LBWT pig skeletal muscle compared with their NBWT siblings. Our current data suggest that the reduction in Sestrin2 expression is a potential compensatory mechanism that should have allowed LBWT pigs to have a greater sensitivity to leucine

While it is not yet clear why BCAA increased translational capacity without having an effect on skeletal muscle protein deposition, leucine supplementation could activate translation initiation without increasing protein synthetic capacity in limit fed neonatal pigs (Boutry et al., 2016; Manjarín et al., 2018). In other studies, in which prolonged leucine supplementation failed to improve lean gain, amino acid availability appeared to limit protein synthesis. When leucine was supplemented to low protein diets of piglets for 20 days, leucine enhanced translation initiation and fractional protein synthesis in longissimus dorsi muscle, but did not improve lean accretion (Columbus et al., 2015). Similarly, neonatal pigs were fed a low protein, low energy formula supplemented with leucine or BCAA for 21 days, translation initiation increased without a corresponding improvement in fractional protein synthesis or muscle growth (Manjarín et al., 2018). Regardless of signaling activation by leucine, all essential amino acids must be supplied in sufficient amounts from the diet for protein synthesis to occur (Escobar et al., 2007; Wilson et

al., 2010). In the current study, diets met all essential amino acid requirements for pigs of that age, and intramuscular concentrations other than BCAA and alanine were similar in all treatment groups, yet translation initiation was not affected by BCAA. One possibility is that amino acid stimulation of mTOR signaling was already maximized by the control diet, making a further enhancement not possible.

Although amino acid availability did not appear to limit lean accretion in the current experiment, it is noteworthy that several nonessential amino acids were reduced in response to BCAA. Circulating concentrations of amino acids are known to decrease in response to leucine supplementation, but their disappearance is credited to increased muscle protein synthesis (Boutry et al., 2016; Escobar et al., 2005; Wilson et al., 2010). There are only two possible causes of circulating amino disappearance in the current study. First, they were oxidized, which was not indicated by plasma urea concentration (Supplemental figure 2.6). Second, they were used for protein accretion, which BCAA did not appear to influence in skeletal muscle. Therefore, amino acids may have gone to protein synthesis elsewhere in the body such as the gastrointestinal tract, which has naturally high rates of protein synthesis and endogenous losses (Nyachoti et al., 1997; Wu et al., 2006). Indeed, oral leucine intake has been shown to increase epithelial proliferation in the duodenum of suckling pigs (Y. Sun et al., 2015) and adult men (Coëffier et al., 2011).

In conclusion, we set out to determine whether oral BCAA supplementation would improve lean tissue accretion in pigs. Our data suggest that although BCAA supplementation ameliorated protein synthetic capacity, there was no improvement in lean tissue accretion or body weight gain of LBWT and NBWT pigs despite a reduction in Sestrin2 expression in skeletal muscle of LBWT pigs. While BCAA supplementation did not enhance lean tissue

accretion, fractional growth rate of LBWT pigs was greater than that of their NBWT siblings, and that was likely due to a more efficient dietary protein utilization.

Table 2.1. Composition of experimental diets (%, as fed).

Item	Diet	
	Control	BCAA
Ingredient		
Whey protein isolate* (90% CP)	6.43	6.43
Lactose*	4.50	4.50
Canola oil	2.69	2.69
Fat Pak 80†	2.38	2.38
Mineral premix†‡	0.90	0.90
Dicalcium phosphate	0.88	0.88
L-Alanine	0.70	-
L-Leucine	-	0.45
L-Isoleucine	-	0.33
L-Valine	-	0.22
Vitamin premix†§	0.20	0.20
L-Histidine	0.10	0.10
Xanthan gum†	0.10	0.10
Calcium carbonate	0.06	0.06
Choline chloride (100%)	0.02	0.02
Water	81.0	80.7
Analyzed nutrient composition		
Dry matter	18.1	18.4
ME, *kcal/kg*#	936	946
Crude protein	6.54	6.63
Crude fat	4.45	4.41
Ash	1.62	1.62
Calcium	0.39	0.42
Phosphorus	0.25	0.27
Calcium : Phosphorus	1.56	1.56
Amino acids		
Alanine	1.02	0.31
Arginine	0.12	0.12
Cysteine	0.15	0.15
Histidine	0.18	0.18
Isoleucine	0.43	0.73
Leucine	0.66	1.09
Lysine	0.59	0.59
Methionine	0.12	0.12
Phenylalanine	0.18	0.18
Threonine	0.44	0.43

Tryptophan	0.14	0.13
Valine	0.38	0.61

Control = Sow milk-replacer formula; BCAA = Control plus 1% branched-chain amino acids;
*Milk Specialties, Eden Prairie, MN.
†Dyets Inc., Bethlehem, PA.
‡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.
§Vitamin premix provided (g/kg): thiamine HCl, 0.1; riboflavin, 0.375; pyridoxine HCl,
ME calculated from measured GE using equations 1-2 and 1-6 (National Research Council, 2012)
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.

Table 2.2. Gene specific primers used for RT-qPCR

Gene	Direction	Primer Sequence	Accession No.
PPIA	Forward	5’ – CGTCTTCTTCGACATCGCCG – 3’	NM_214353.1
	Reverse	5’ – GAAGTCACCACCCTGGCACATAAAT – 3’	
H3F3A	Forward	5’ – GGTAAGTAAGGAGGTCTCTATACCA – 3’	NM_213930.1
	Reverse	5’ – TAATTTCACGGAGTGCCACG – 3’	
B2M	Forward	5’ – TTCAGGTTTACTCACGCCAC – 3’	NM_213978.1
	Reverse	5’ – GAACTCAGTGTGGACCAGAAGG - 3’	
RNF7	Forward	5’ – TGGGACGTGGAGTGCGATAC – 3’	NM_001245011.1
	Reverse	5’ – CATGCAGCAGTTGTGGAAGGA – 3’	
BCAT2	Forward	5’ – CCT CTT CCG ACC TTG GCT CA – 3’	XM_013998608.2
	Reverse	5’ – ACC GGG CAG AGG ATG ACG TA – 3’	
BCKDHA	Forward	5’ – TGG ACC GCA TCC TCT ACG AG – 3’	NM_001123083.1
	Reverse	5’ – AGG TCG CTC ACG TTG CTG TA – 3’	
IGF1	Forward	5′ – GCA CAT CAC ATC CTC TTC GC – 3′	NM_214256.1
	Reverse	5′ – ACC CTG TGG GCT TGT TGA AA – 3′	
IGF1R	Forward	5′ – CAT ACC AGG GCT TGT CCA AC – 3′	NM_214172.1
	Reverse	5′ – ATC AGC TCA AAC AGC ATG TCG – 3′	

Table 2.3. Body weight gain, average food intake, and food efficiency of low (L) and normal (N) birthweight pigs fed a control (C) or a branched-chain amino acid supplemented (B) diet for 28 days [1]

	Treatment					*P*-value		
	LC	NC	LB	NB	SEM	Birthweight	Diet	Birthweight × Diet
Body weight gain, *kg* [2]	4.7	5.6	4.6	5.7	0.37	<0.01	0.98	0.76
Food intake, *L*	19	25	18	26	1.52	<0.0001	0.98	0.65
Food efficiency, $kg \bullet L^{-1}$	0.25	0.22	0.25	0.22	0.004	<0.0001	0.88	0.52

[1] Pigs received 250 mL· kg body weight $^{-1}$ · d^{-1} in 5 equal meals every 4 hours from 8:00am to 12:00am.
[2] Values are least square means. SEM, Standard Error of the Means, *n* = 5-7. Food efficiency was calculated as kilogram body weight gained per liter of formula consumed.

Table 2.4. Fat gain, lean gain, and the efficiency of energy and protein deposition of low (L) and normal (N) birthweight pigs fed a control (C) or a branched-chain amino acid supplemented (B) diet for 28 days

	Group					*P*-value		
	LC	NC	LB	NB	SEM	Birthweight	Diet	Birthweight × Diet
Fat accretion, *kg*	0.34	0.45	0.30	0.50	0.058	<0.05	0.97	0.47
Lean accretion, *kg*	4.8	5.4	4.6	5.6	0.34	<0.05	0.92	0.60
Fat accretion, *% of gain*	6.5	7.6	5.9	7.8	0.64	<0.05	0.79	0.60
Lean accretion, *% of gain*	93	91	93	91	0.76	<0.05	0.75	0.58
Efficiency of energy retention, *%*	47	44	46	44	2.0	0.20	0.73	0.88
Efficiency of protein retention, *%*	75	66	75	65	1.6	<0.0001	0.67	0.54

Results are least square means and SEM, n = 5-7.

Table 2.5. Organ and muscle weights of low (L) and normal (N) birthweight pigs fed a control (C) or a branched-chain amino acid supplemented (B) diet for 28 days[1]

	Group					*P*-value		
	LC	NC	LB	NB	SEM	Birthweight	Diet	Birthweight × Diet
Final body weight, *kg*	6.4	7.8	6.4	8.1	0.46	<0.001	0.83	0.70
Tissue weight, *g*								
Liver	235	287	229	287	19.1	<0.0001	0.67	0.66
L. dorsi	108	132	106	150	11.1	<0.01	0.50	0.40
Soleus	4.2	5.5	4.1	5.3	0.52	<0.001	0.56	0.94
Semitendinosus	19	22	16	25	2.2	<0.01	0.97	0.08
Tissue weight, *% body weight*								
Liver	3.8	3.6	3.7	3.6	0.11	0.33	0.66	0.36
L. dorsi	1.7	1.7	1.6	1.8	0.09	0.20	0.57	0.47
Soleus	0.067	0.069	0.064	0.066	0.0039	0.53	0.45	0.87
Semitendinosus	0.30	0.28	0.25	0.30	0.023	0.47	0.50	0.06

Values are least square means and SEM, n = 5-7.

Table 2.6. Longissimus dorsi amino acid concentration of low (L) and normal (N) birthweight pigs fed a control (C) or a branched-chain amino acid supplemented (B) diet for 28 days

	Group					*P*-value		
Amino acid	LC	NC	LB	NB	SEM	Birthweight	Diet	Birthweight × Diet
			μmol • g^{-1}					
Essential								
Histidine	0.22	0.21	0.22	0.20	0.018	0.23	0.81	0.69
Isoleucine	0.40	0.42	0.68	0.64	0.051	0.85	<0.0001	0.67
Leucine	0.47	0.51	0.95	0.89	0.071	0.89	<0.0001	0.48
Lysine	1.3	1.1	1.0	1.3	0.15	0.72	0.44	0.08
Methionine	0.076	0.071	0.055	0.057	0.0120	0.88	0.13	0.74
Phenylalanine	0.045	0.065	0.047	0.043	0.0075	0.18	0.10	0.08
Threonine	3.4	3.1	3.0	3.4	0.31	0.76	0.75	0.21
Valine	0.54	0.63	1.16	1.13	0.085	0.71	<0.0001	0.44
Non-essential								
Alanine	4.1	3.5	2.3	2.3	0.17	0.12	<0.0001	0.05
Aspartate	0.48	0.48	0.48	0.49	0.056	0.92	0.94	0.89
Glutamate	2.8	3.1	2.4	2.8	0.40	0.18	0.16	0.76
Glutamine	11	11	12	12	1.3	0.95	0.20	0.84
Glycine	4.6	4.6	4.6	5.5	0.35	0.13	0.17	0.12
Proline	1.5	1.7	1.6	1.8	0.22	0.44	0.49	0.87
Serine	1.4	1.3	1.3	1.3	0.09	0.31	0.83	0.86
Tyrosine	0.079	0.088	0.071	0.071	0.0079	0.52	0.12	0.58

Table 2.7. Total RNA, protein and protein synthetic capacity in longissimus dorsi of low (L) and normal (N) birthweight pigs fed a control (C) or a branched-chain amino acid supplemented (B) diet for 28 days

	Group				SEM	*P*-value		
	LC	NC	LB	NB		Birthweight	Diet	Birthweight × Diet
RNA, *mg • g*$^{-1}$	1.6	1.7	1.8	1.9	0.09	0.33	<0.05	1.00
Protein, *mg •g*$^{-1}$	76	77	77	76	3.4	0.89	0.97	0.71
Protein synthetic capacity, *µg RNA • mg protein*$^{-1}$	21	23	25	25	1.8	0.37	<0.05	0.75

Values are least square means and SEM, *n* = 5-7. Protein synthetic capacity = mg RNA • g protein^{-1}

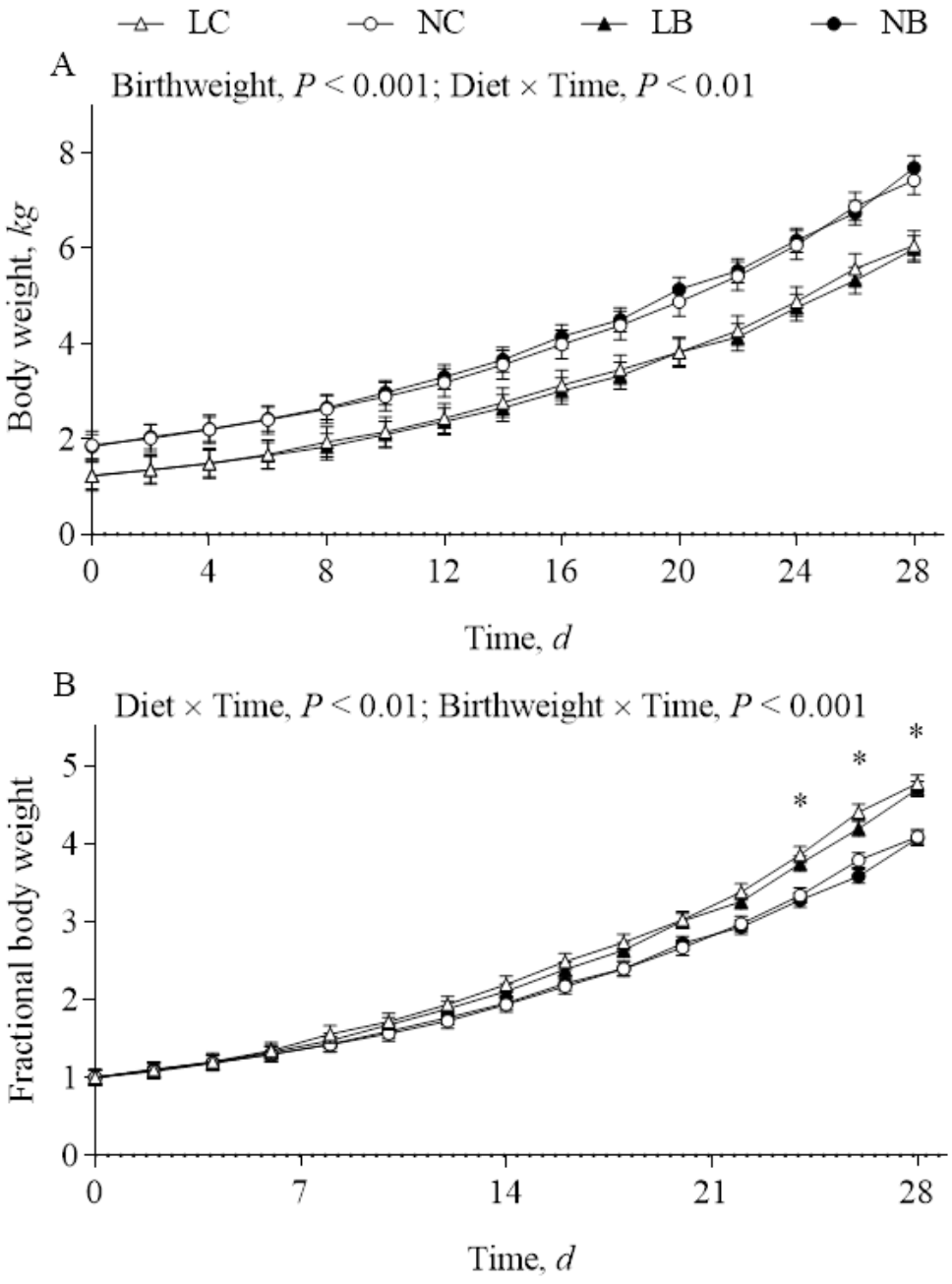


Figure 2.1. Body weight (A) and fractional body weight (B) over time of low (L) and normal (N) birthweight pigs fed a control (C) or a branched-chain amino acid supplemented (B) diet for 28 d. Results are least square means and SEM, *n* = 5-7.

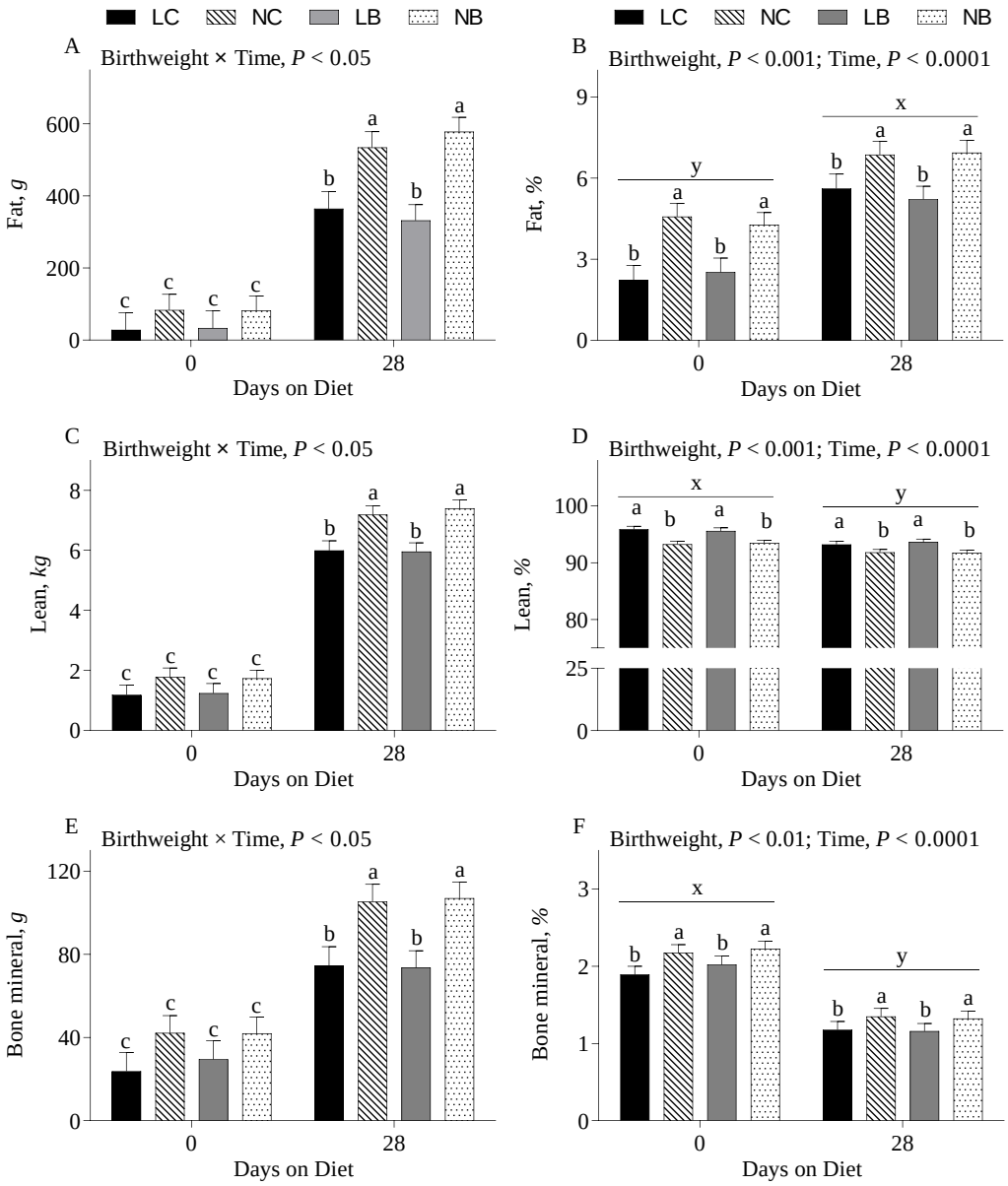


Figure 2.2. Body composition of low (L) and normal (N) birthweight pigs fed a control (C) or a branched-chain amino acid supplemented (B) diet for 28 d, measured by DXA at beginning and end of study. Results are least square means ± SEM, n = 5-7. Means without a common letter differ by birth weight, irrespective of time, P ≤ 0.05.

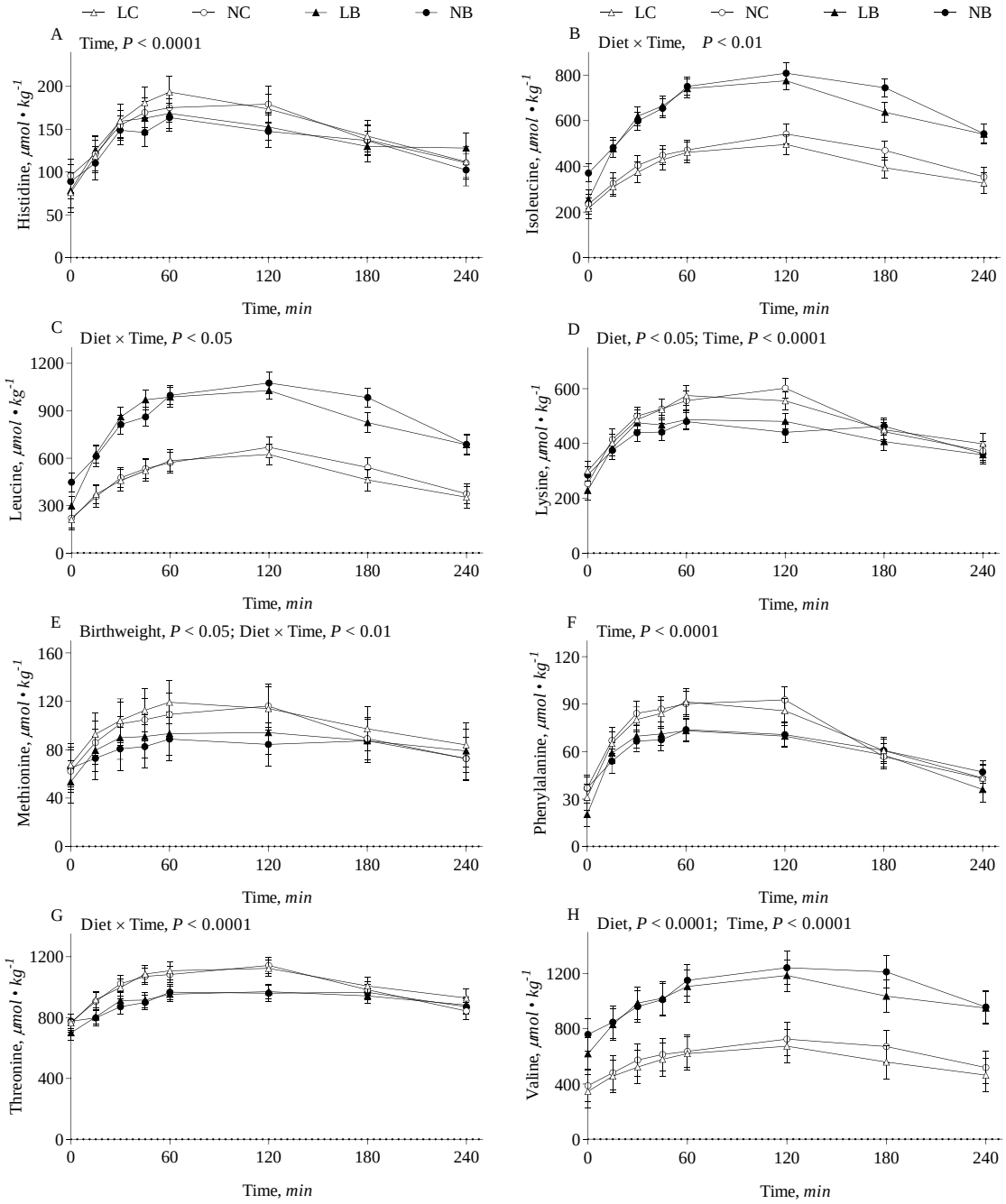


Figure 2.3. Essential amino acid concentrations in blood plasma over 4 hours following a meal in low (L) and normal (N) birthweight pigs fed a control (C) or a branched-chain amino acid supplemented (B) diet for 28 d. Results are least square means ± SEM, n = 5-7.

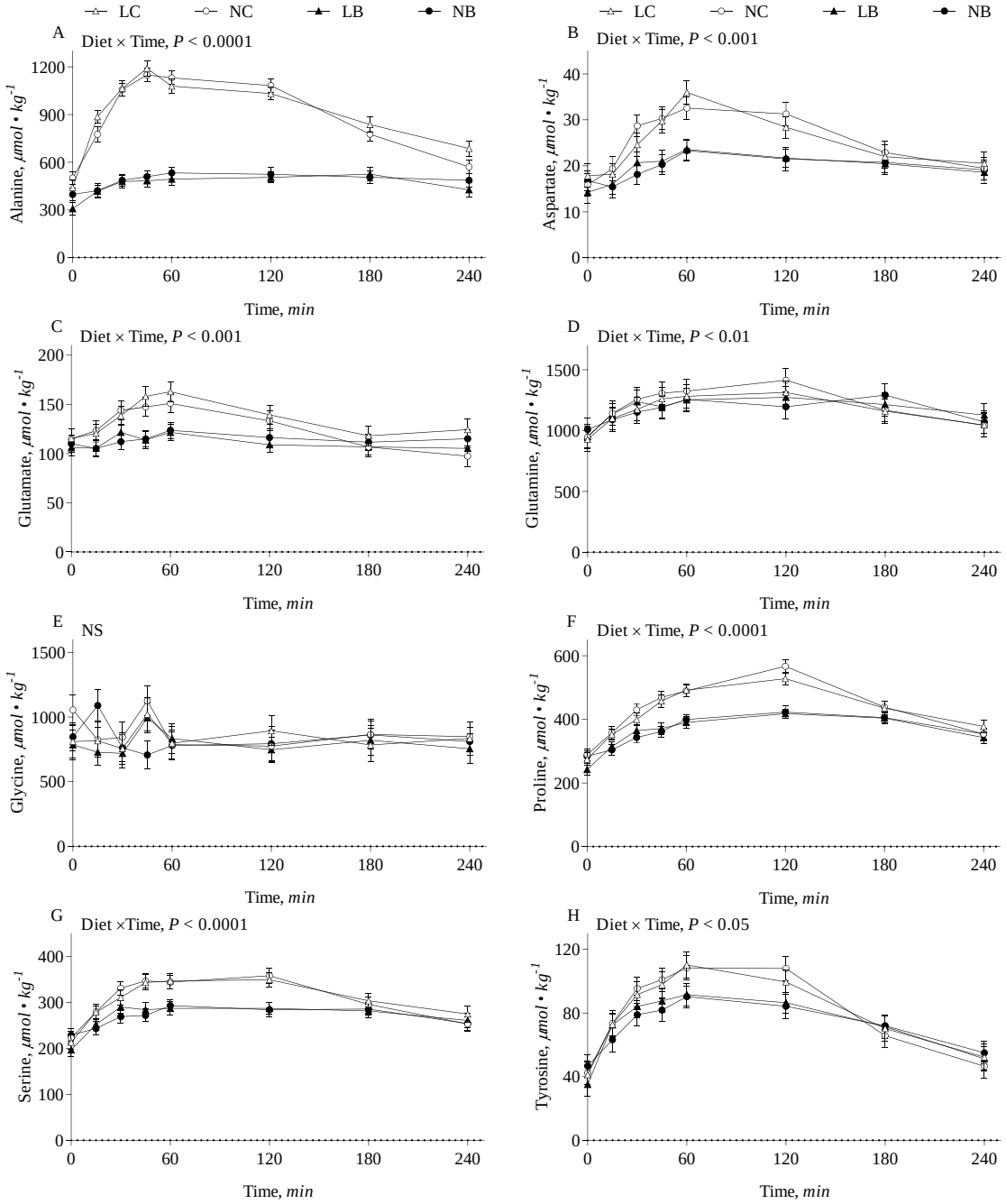


Figure 2.4. Nonessential amino acid concentrations in blood plasma over 4 hours following a meal in low (L) and normal (N) birthweight pigs fed a control (C) or a branched-chain amino acid supplemented (B) diet for 28 d. Results are least square means ± SEM, n = 5-7.

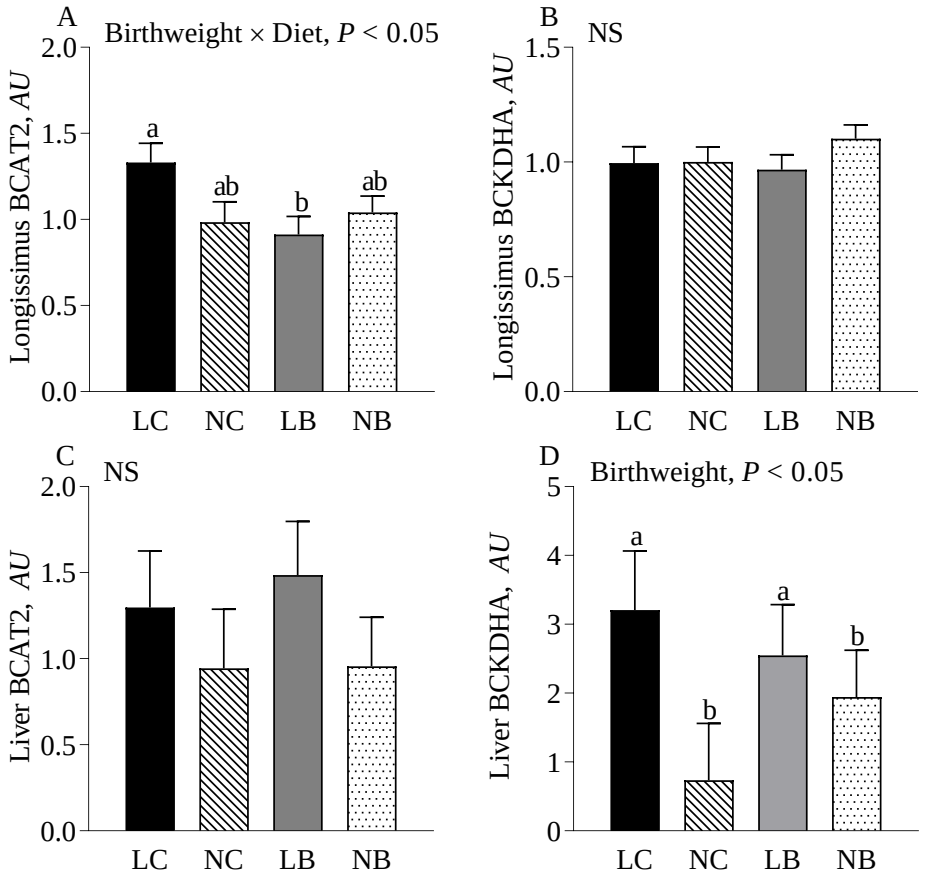


Figure 2.5. Relative mRNA expression of BCAA catabolizing enzymes in the longissimus dorsi and liver of low (L) and normal (N) birthweight pigs fed a control (C) or a branched-chain amino acid supplemented (B) diet for 28 d. Results are least square means ± SEM, n = 5-7. Means without a common letter differ by the specified main effect or interaction, $P \leq 0.05$.

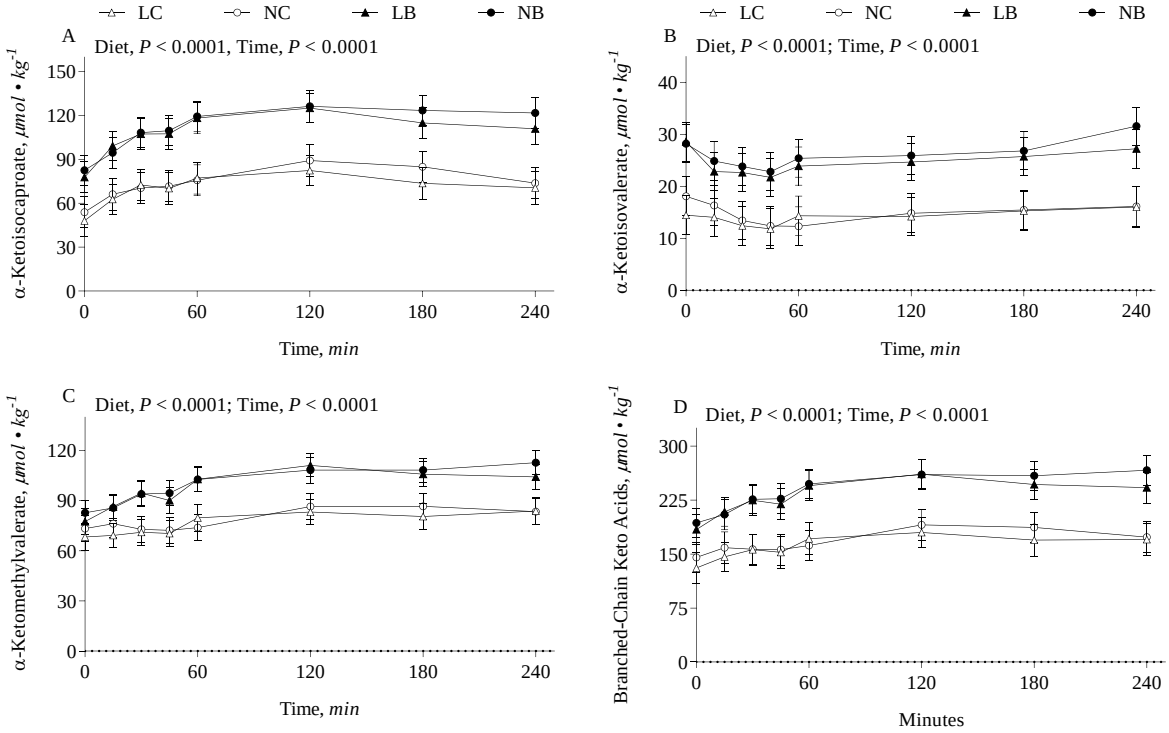


Figure 2.6. Branched-chain α-keto acid concentrations in blood plasma over 4 hours following a meal in low (L) and normal (N) birthweight pigs fed a control (C) or a branched-chain amino acid supplemented (B) diet for 28 d. Results are least square means ± SEM, n = 5-7.

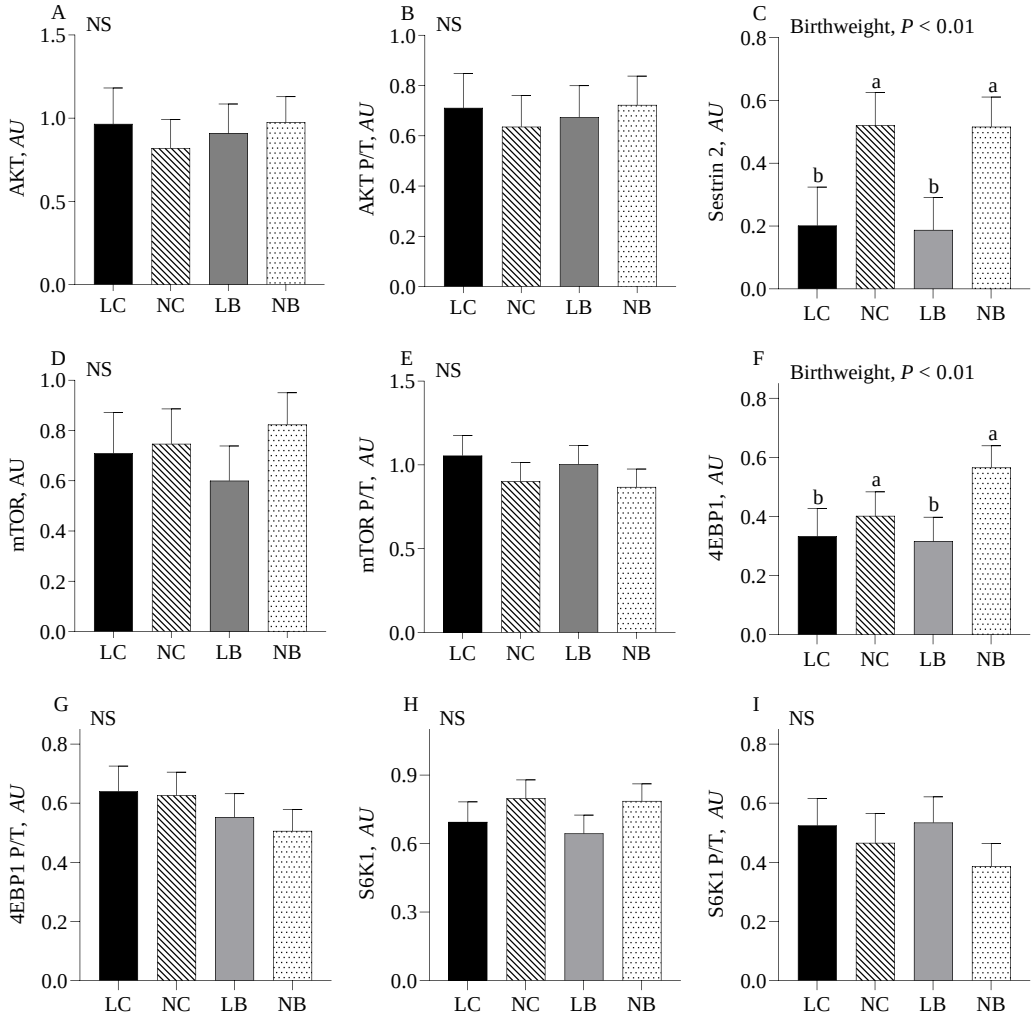
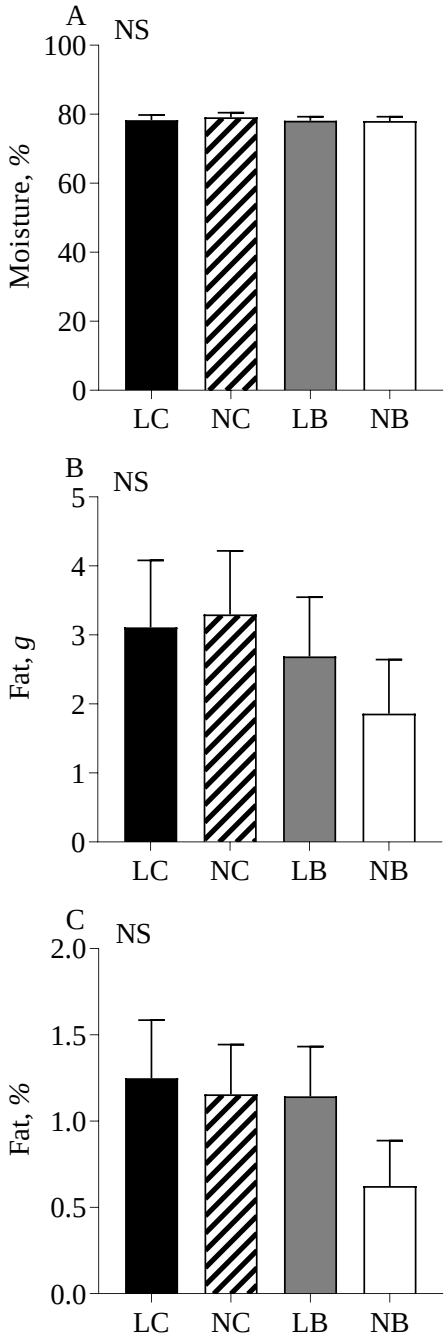


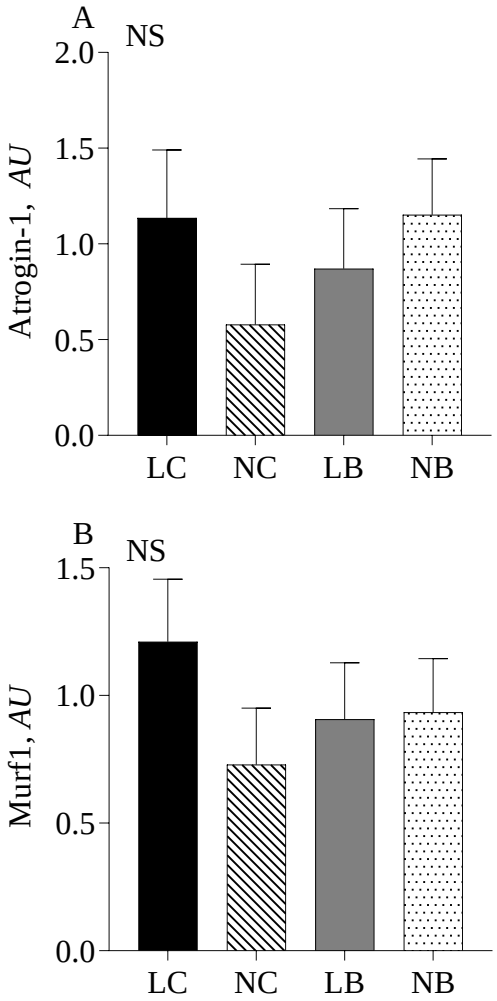
Figure 2.7. Expression and phosphorylation of translation initiation regulating proteins in longissimus dorsi of low (L) and normal (N) birthweight pigs fed a control (C) or a branched-chain amino acid supplemented (B) diet for 28 d. Results are least square means ± SEM, n = 5-7. Means without a common letter differ by the specified main effect or interaction, $P \leq 0.05$.

Supplemental Table 2.1. Protein specific antibodies used for western blot applications

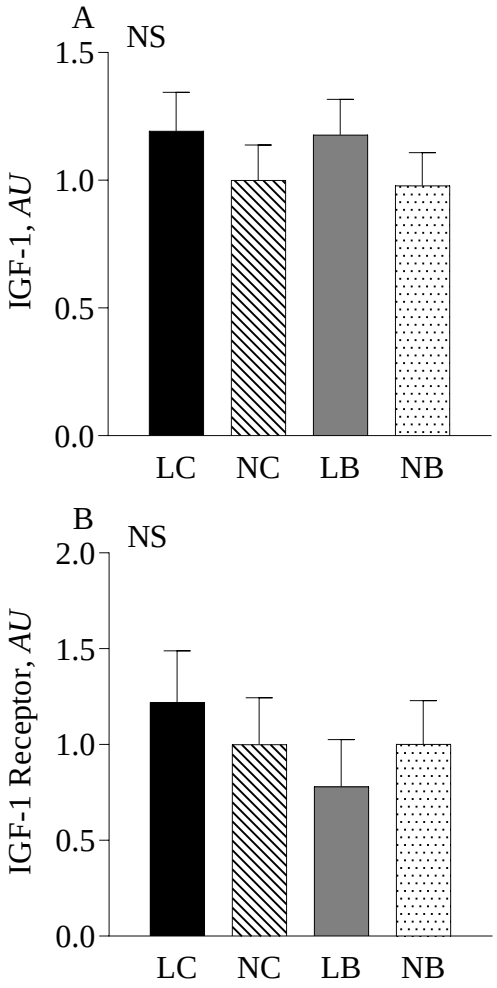
Antibody	Company
α-Tubulin	#2144; Cell Signaling Technology, Danvers, MA
Akt	#9272; Cell Signaling Technology, Danvers, MA
Phospho-Akt (Ser^{473})	#9271; Cell Signaling Technology, Danvers, MA
Sestrin-2	#8487; Cell Signaling Technology, Danvers, MA
eIF3e	#AB1114851; Abcam, Cambridge, MA
mTOR	#2983; Cell Signaling Technology, Danvers, MA
Phosph-mTOR (Ser^{2481})	#2974; Cell Signaling Technology, Danvers, MA
P70 S6 kinase	#49D7; Cell Signaling Technology, Danvers, MA
Phosph-S6K1 (Thr^{389})	#07-018-I; Millipore, Temecula, CA
4EBP1	#A300-501A; Bethyl Laboratories, Montgomery, TX
Phospho-4EBP1(Thr^{46})	#441170; Invitrogen, Camarillo, CA



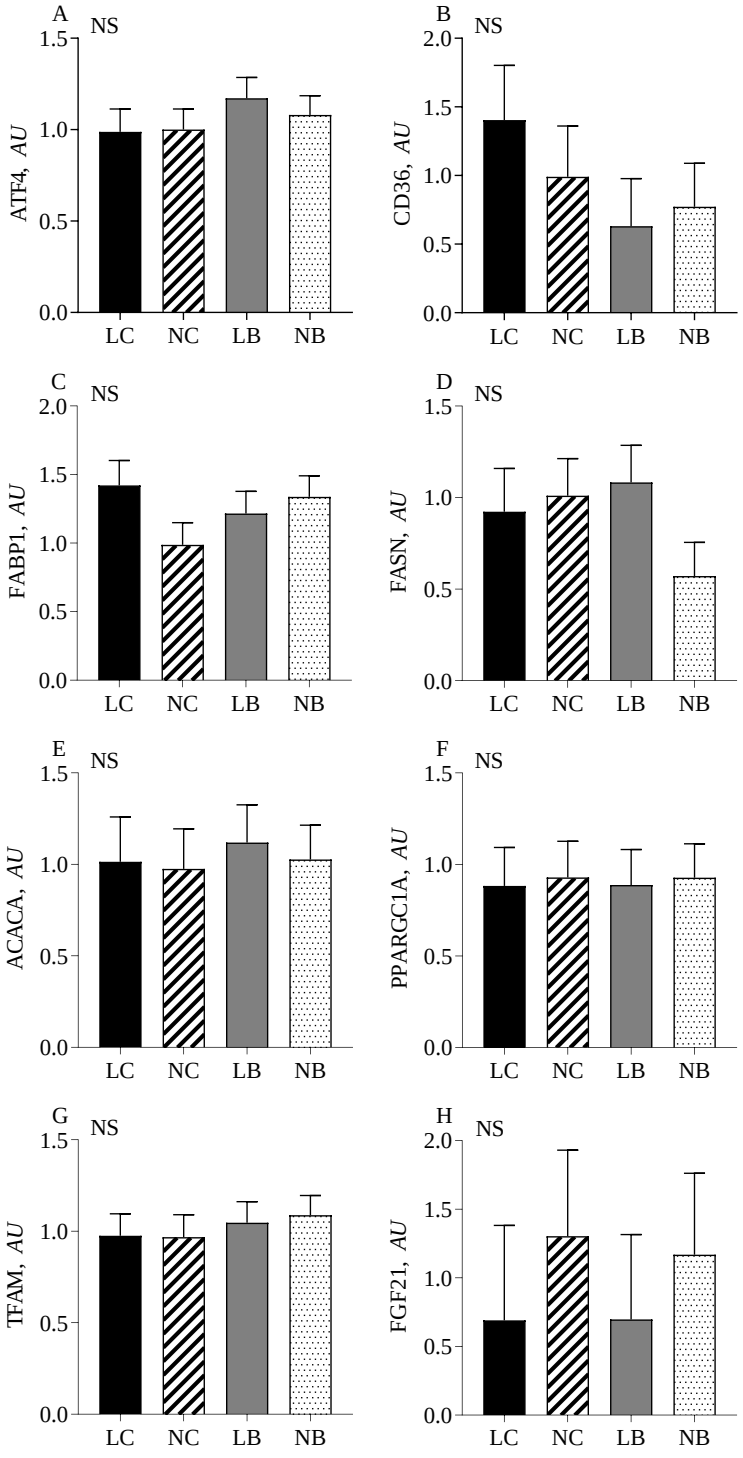
Supplemental Figure 2.1. Moisture and fat content of livers of low (L) and normal (N) birthweight pigs fed a control (C) or a branched-chain amino acid supplemented (B) diet for 28 d. Results are least square means ± SEM, n = 5-7.



Supplemental Figure 2.2. Relative protein expression of muscle specific ubiquitin ligases in longissimus dorsi of low (L) and normal (N) birthweight pigs fed a control (C) or a branched-chain amino acid supplemented (B) diet for 28 d. Results are least square means ± SEM, n = 5-7.



Supplemental Figure 2.3. Relative mRNA expression of IGF-1 and IGF-1 receptor in longissimus dorsi of low (L) and normal (N) birthweight pigs fed a control (C) or a branched-chain amino acid supplemented (B) diet for 28 d. Results are least square means ± SEM, n = 5-7.


A
NS
ATF4, AU
1.5
1.0
0.5
0.0
LC
NC
LB
NB
B
NS
CD36, AU
2.0
1.5
1.0
0.5
0.0
LC
NC
LB
NB
C
NS
FABP1, AU
2.0
1.5
1.0
0.5
0.0
LC
NC
LB
NB
D
NS
FASN, AU
1.5
1.0
0.5
0.0
LC
NC
LB
NB
E
NS
ACACA, AU
1.5
1.0
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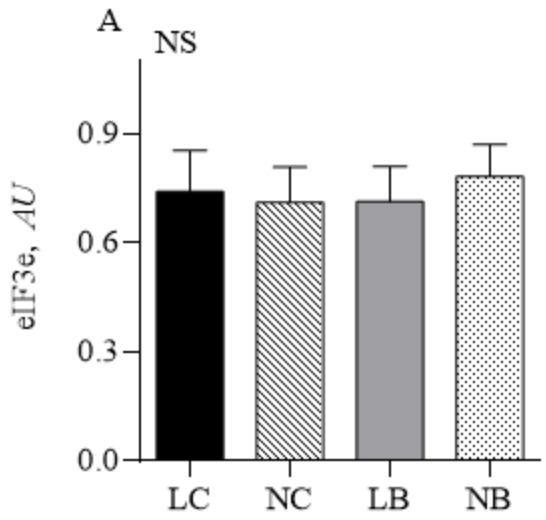
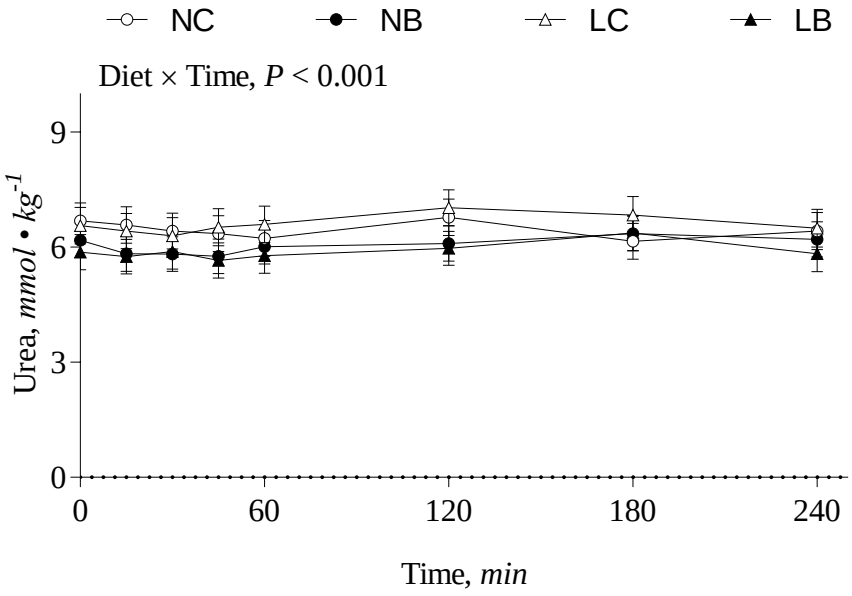
0.0
LC
NC
LB
NB
F
NS
PPARGC1A, AU
1.5
1.0
0.5
0.0
LC
NC
LB
NB
G
NS
TFAM, AU
1.5
1.0
0.5
0.0
LC
NC
LB
NB
H
NS
FGF21, AU
2.0
1.5
1.0
0.5
0.0
LC
NC
LB
NB

Supplemental Figure 2.4. Relative mRNA expression of genes regulating energy expenditure (A, F, and H), fatty acid transport (B and C), and fatty acid synthesis (D and E) in livers of low (L) and normal (N) birthweight pigs fed a control (C) or a branched-chain amino acid supplemented (B) diet for 28 d. Results are least square means ± SEM, n = 5-7.



Supplemental Figure 2.5. Relative protein expression of eIF3e in longissimus dorsi of low (L) and normal (N) birthweight pigs fed a control (C) or a branched-chain amino acid supplemented (B) diet for 28 d. Results are least square means ± SEM, n = 5-7.



Supplemental Figure 2.6. Urea concentration in blood plasma over 4 hours following a meal in low (L) and normal (N) birthweight pigs fed a control (C) or a branched-chain amino acid supplemented (B) diet for 28 d. Results are least square means ± SEM, n = 5-7.

Chapter 3. High MCFA Diets Cause Hepatic Fat and Amino Acid Metabolic Dysfunction

Abstract

Plasma concentrations of branched-chain amino acids (BCAA) are elevated during obesity and associated conditions. We have previously demonstrated that BCAA supplementation does not lead to dysfunction in hepatic fat metabolism. The aim of the current experiment was to demonstrate whether high fat diets consisting of long-chain (LCFA) or medium-chain fatty acids (MCFA) elevate plasma BCAA by disturbing BCAA catabolism. Neonatal pigs were fed milk replacement formula supplying 80% of NRC protein requirements and either 80% of energy (Control) or 120% of energy requirement from animal fat (LCFA) or coconut oil (MCFA) for 22 days. Body composition was measured by dual-energy X-ray absorptiometry on day 0 and 18 of feeding. An intravenous glucose tolerance test was performed on day 18, and whole-body rate of leucine appearance was measured on day 21 of feeding by primed, continuous infusion of [1-^{13}C] leucine. Pigs fed the high fat diets had greater body fat percentage ($P < 0.0001$) and their livers weighed more ($P<0.05$) compared to those fed the Control diet. MCFA increased the rate of plasma glucose removal in an IVGTT ($P<0.0001$) and decreased postprandial rate of leucine appearance ($P<0.0001$) compared to the other diets. MCFA also caused increased plasma α-keto isovalerate (KIV) ($P<0.05$) compared to Control, and KIV concentration resulting from LCFA was intermediate. Plasma glutamate concentration was greater for pigs fed the MCFA than those fed the LCFA or Control diets ($P<0.0001$). Less KIV was oxidized to CO_2 in liver homogenate of MCFA fed pigs than those fed the Control diet ($P<0.05$), with an intermediate amount oxidized by LCFA pigs, and both high fat diets decreased oxidation of α-ketoisocaproate compared to Control ($P<0.001$). Diet did not affect expression of

BCAA catabolizing genes, but MCFA greatly increased hepatic ACACA and FASN expression compared to Control and LCFA ($P<0.01$). Our data demonstrate that high MCFA diets cause liver specific fat-related dysfunction and decrease branched-chain α-keto acid oxidation.

Introduction

Elevated concentrations of plasma branched-chain amino acids (BCAA) in obese humans was first reported over 50 years ago (Felig, 1970), but the relationship between obesity and BCAA is still not clear. BCAA are a group of non-polar, methylated, essential amino acids consisting of valine, leucine, and isoleucine. Recently, the use of BCAA gained popularity as dietary supplements and are the focus of much research due to their anabolic signaling properties (Crozier et al., 2005; Escobar et al., 2006; Murgas Torrazza et al., 2010). Therefore, it is important to understand whether BCAA contribute to obesity or metabolic dysfunction in order to inform supplementation and consumption.

In non-obese animals, BCAA are beneficial for preventing adiposity (Freudenberg et al., 2012; H. Li et al., 2012b; Vianna et al., 2012) and improving glucose homeostasis (Binder et al., 2013; Eller et al., 2013; Zhang et al., 2007). We have previously reported that high BCAA consumption did not affect adiposity or hepatic mRNA expression related to fat or energy metabolism in neonatal pigs (Yonke et al., 2020). However, BCAA have exacerbated insulin resistance in obese rats (Newgard et al., 2009) and in mice with nonalcoholic fatty liver disease (Sunny et al., 2015), suggesting BCAA contribute to metabolic dysfunction in excessively fatty conditions.

The current evidence suggest that fasting plasma BCAA concentrations are greater in obese (Felig, 1970; Newgard et al., 2009) and insulin resistant humans (Tan et al., 2018) than in normal individuals. Others have also suggested that plasma BCAA are a predictive marker of type II diabetes (T. J. Wang et al., 2011). Although reports that genetically obese and insulin

resistant rodents (Maida et al., 2017; She et al., 2007; Zhou et al., 2019) have elevated plasma BCAA, the relationship between diet induced obesity and BCAA dysregulation were inconsistent. Models of diet induced obesity did not elevate plasma BCAA in rodents (J. Lee et al., 2021b; Newgard et al., 2009; P. J. White et al., 2016), but in pigs, high fat, high sugar diets increased hepatic (Frano et al., 2019) and plasma (Polakof et al., 2018) BCAA concentrations. A neonatal pig model was chosen for the current experiment due to their metabolic similarity to humans (Ferenc et al., 2014) and ease of jugular/carotid catheterization.

We have previously reported that long-term BCAA supplementation increases plasma BCAA in neonatal pigs but does not exacerbate adiposity or alter hepatic fat metabolism (Yonke et al., 2020). Therefore, the aim of this work was to investigate whether hepatic fat metabolism precedes BCAA metabolic dysregulation using a dietary neonatal model of liver steatosis. The hypothesis of this study was that high fat diets would alter BCAA metabolism in neonatal pigs. Thus, the objectives of this study were to 1) determine the effect of dietary fat on whole body leucine flux, and 2) investigate the regulation of hepatic BCAA catabolism in response to feeding high fat diets.

Materials and Methods

Animals and surgeries. All animal procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee. White crossbred pigs were selected for similar birth weight and equal number of boars and gilts. At two day of age, 24 pigs were pair-housed in cages and trained to drink milk-replacer formula from troughs. Pigs were kept in rooms maintained at 27°C and supplemented with heat lamps which were removed at 14 days of age. All pigs were fed the Control diet during a three-day adaptation period before surgical catheter

placement. Pigs were fasted overnight before fitting with indwelling catheters (AAQ0427, Tygon Company) in the carotid artery and jugular vein using sterile techniques under general isoflurane anesthesia as previously described (El-Kadi et al., 2019). Pigs were moved to individual cages and allowed seven days to recover before being assigned to experimental diets. Seven pigs were removed during the study due to loss of catheter patency or loss of appetite, resulting in Control (n=5, 2 boars, 3 gilts), LCFA (n=6, 3 boars, 3 gilts), and MCFA (n=6, 4 boars, 2 gilts). Pigs were killed on day 22 of the study for tissue collection 60 minutes after their first meal of the day.

Diets. Experimental milk-replacer formulas were formulated to meet 80% of NRC protein requirements and either 80% of energy (Control) (Table 3.1) or 120% of energy requirement from an animal fat blend rich in long-chain fatty acids (LCFA) or from virgin coconut oil rich in medium-chain fatty acids (MCFA). Milk was mixed fresh every-other day and refrigerated between feedings. All pigs were provided milk at 250mL · kg of body weight^{-1} · d^{-1}, divided among 5 meals fed every 4 h (0800, 1200, 1600, 2000, and 0000) for 22 days. Pigs were weighed every-other day and feed allotment was adjusted accordingly.

Dual-energy X-ray absorptiometry. Body composition was measured by dual-energy X-ray absorptiometry (DXA) (Lunar Prodigy; GE Medical systems, Milwaukee, WI) using Small Animal Body scan (Nord et al., 2001). Pigs were fasted overnight and lightly sedated by isoflurane for scans on the morning of days 0 and 18 of the study. Tissue mass on day 22 was estimated by multiplying day 22 body weights by day 18 tissue percentages (El-Kadi et al., 2019). Protein deposition was calculated using the formula (Mitchell et al., 1998)

$$\text{Body protein (g)} = -1.062 + (0.2 \cdot \text{DXA lean})$$

Energy deposition was calculated assuming 5,680 and 9,460 kcal • kg^{-1} as the caloric content of protein and fat (El-Kadi, Boutry, et al., 2018). Efficiency of energy and protein retention were calculated from energy and protein retained and total dietary energy and protein consumed over the duration of the study (El-Kadi, Boutry, et al., 2018).

Intravenous-glucose-tolerance test. Following an overnight fast on day 18 of the study, glucose clearance was measured by an intravenous-glucose tolerance test (IVGTT). A fasting arterial blood sample was taken into a heparinized syringe. A bolus dose of dextrose (1 mg • kg^{-1} of body weight) was injected into the venous catheter over 1 min, immediately followed by arterial blood sample collection at 1, 2, 4, 8, 15, 30, 45, and 60 m. Plasma was separated from heparinized blood by centrifugation at 4°C for 10 min at 10,000 × g.

Tracer infusion and blood sampling. On day 21 of the study [1-^{13}C] leucine (Cambridge Isotope Laboratories) was infused into the venous catheter at 10 μmol•kg^{-1}•h^{-1}. A blood sample was taken from each pig before infusion to measure background leucine enrichment. Tracer infusion began at the time of feeding the first meal of the day and lasted 8 h. A blood sample was taken 4 h after beginning the infusion, followed by feeding the next meal. Blood was drawn at 15, 30, 45, 60, 120, 180, and 240 minutes after the meal.

Amino acid, branched-chain alpha-keto acid, urea, and glucose concentration. A known weight of plasma was combined with a mix of internal standards including [U-^{13}C, ^{15}N] amino acids and [$^{15}N_2$] urea (Cambridge Isotope Laboratories, Inc, Andover, MA), and α-ketovaleric acid (Sigma-Aldrich Co., St. Louis, MO) and were combined and stored at -20°C until further analysis. Internal standards were similarly added to longissimus dorsi samples and homogenized in sulfosalicylic acid (8% final concentration) prior to extraction. Free amino acids, urea, and branched-chain α-keto acids (BCKA) were prepared as previously described (Lobley et al.,

2003) and quantified by gas chromatography/mass spectrometry (GC 7890 with 5975 mass selective detector; Agilent Technologies, Wilmington, DE) on a 30 m x 0.25 mm x 0.25 μm HP-5MS fused silica capillary column (Agilent Technologies, Wilmington, DE). Glucose concentration in plasma was also analyzed by gas chromatography/mass spectrometry as previously described (Hannestad & Lundblad, 1997) using [U-$^{13}C_6$]-glucose (Cambridge Isotope Laboratories, Inc, Andover, MA) as internal standard and a 30 m x 0.25 mm x 0.25 μm DB-17 column (Agilent Technologies, Wilmington, DE).

Calculations. When isotopic steady state was reached 4 h after initiation of [1-^{13}C] leucine infusion and a meal, the rate of appearance (Ra) of leucine was calculated using the following equation:

$$Ra = (E_t/E_A - 1) \times IR$$

where Et is the enrichment of the leucine tracer infused, EA is the arterial enrichment of leucine, and IR is the infusion rate (in $mmol \cdot kg^{-1} \cdot h^{-1}$) of the leucine tracer. After a meal, Ra was calculated according to a modified Steele's equation (Proietto et al., 1987) for non–steady state conditions:

$$Ra = IR - pVd \times Cm(t) \times dE/dt / Em(t)$$

where IR is the infusion rate ($\mu mol \cdot kg^{-1} \cdot h^{-1}$) of leucine tracer, p is the pool fraction for instant mixing (p= 0.25) (Boirie et al., 1996), Vd is the volume of distribution (Vd = 0.5 L/kg body weight), Cm(t) is the mean arterial leucine concentration ($\mu mol \cdot L^{-1}$), dE/dt is the change in leucine enrichment between successive time points, and Em(t) is the mean enrichment of leucine between 2 time points.

Quantitative real-time PCR. RNA was extracted from the longissimus dorsi muscle and liver using Direct-zol RNA Miniprep kit (ZYMO Research, Orange, CA). First strand cDNA

was generated from RNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Samples were mixed with primers (Table 3.2) and Fast SYBR Green (Applied Biosystems, Foster City, CA) and run in triplicate on 96-well plates using an ABI 7500 Fast Real Time PCR system (Applied Biosystems, Foster City, CA). All primers were validated by agarose gel electrophoresis, amplification efficiency curve, and product sequencing. mRNA abundance relative to the Control group was quantified using the $2^{-\Delta\Delta Ct}$ method relative to the geometric mean of H3F3A and TOP2B in the longissimus dorsi and of TBP and B2M in the liver

Western blot. Abundance and phosphorylation of signaling proteins was performed by SDS-PAGE followed by immunoblotting using specific antibodies as previously described (Y. Chen, McCauley, et al., 2017). Abundance of α-Tublin was used as a loading control, and the phosphorylated proteins were normalized to their corresponding non-phosphorylated form.

Substrate oxidation. Substrate oxidation in liver tissue was analyzed using radio-labeled tracers as previously described (Specht et al., 2021). Briefly, branched-chain alpha-keto acid oxidation was assessed using [1-^{14}C]-α-ketoisovaleric acid (KIV) and [1-^{14}C]-α-ketoisocaproic acid (KIC) to quantify $^{14}CO_2$ production. Liver samples were homogenized and incubated separately in 200 µM [1-^{14}C]-KIV or [1-^{14}C]-KIC (American Radiolabeled Chemicals, St. Louis, MO) for 1 h then acidified with 200µL of 45% perchloric acid 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, Indianapolis, IN). The total protein content in the liver homogenates was measured via a bicinchoninic acid procedure (Thermo Fisher Scientific, Waltham, MA) and used to normalize the oxidation values.

Statistical analysis. Data were analyzed by one-way ANOVA using the PROC MIXED procedure in SAS version 9.3 (SAS Institute Inc.). Temporal effects were investigated using repeated-measures analyses. Various autocorrelation structures were tested, and first-order autoregressive covariance structure gave the best fit based on the Bayesian Information Criterion and was used for analysis of growth, DXA, and IVGTT data. For postprandial plasma concentrations of amino acids and glucose, compound symmetry gave the best fit and was used for analysis. For all comparisons, diet, time, and diet by time interaction were fixed effects, and sow and sex were random effects. When a significant treatment effect was detected, means were compared using the Tukey–Kramer multiple comparison test. The main effects of diet and/or time are presented on graphs. Only diet by time interaction effect was reported when the interaction effect was significant. Data are expressed as the least squares means ± SEMs, and differences are considered significant at $P \leq 0.05$.

Results

Animal Growth. After 22 d of feeding, body weight of pigs fed the three formulas was not different (Figure 3.1). Lean tissue accretion increased over the feeding period but was similar for all groups (Figure 3.2). Moreover, fat mass was similar for pigs fed the control diet at the beginning and end of feeding period but was greater for pigs fed the LCFA and MCFA diets compared with those fed the Control ($P < 0.001$). Gain to feed ratio was greater for pigs fed the LCFA formula compared with those fed the Control or MCFA formulas ($P < 0.02$). Energy accretion was greater for pigs fed LCFA compared with those fed the Control diet with those fed MCFA being intermediate (Table 3.3). Efficiency of energy and protein retention was similar for all groups (Table 3.3).

There were no difference in muscle and organ weights except for liver weight (Table 3.4) which were greater (90g) for pigs fed the MCFA and LCFA formulas compared with those in the Control group ($P < 0.05$). Similarly, when expressed as a percentage of body weight, liver weight was greater (4.7 and 5.2%) for pigs fed the LCFA and MCFA formulas compared with those in the Control (3.6%) group ($P < 0.01$). In addition, kidney weight as a percent of body weight was greater for pigs fed the MCFA (0.43%) than those fed LCFA or Control (0.35%; $P < 0.001$).

Nutrients and Metabolites. Pigs fed MCFA cleared plasma glucose faster during an IVGTT (Figure 3.3) compared with Control and LCFA ($P < 0.0001$). However, fasting and fed plasma glucose concentrations were not affected by diet (Figure 3.4).

The MCFA diet reduced whole-body leucine appearance rate (Figure 3.4) following a meal ($P < 0.0001$). Diet had no effect on postprandial plasma BCAA concentrations (Figure 3.6). Postprandial concentrations of KIV (Figure 3.7), however, were increased by MCFA compared to Control ($P < 0.05$) although plasma concentrations of other BCKA were not significantly affected by diet. Plasma alanine (Figure 3.8) and tryptophan (Figure 3.6) concentrations were decreased by MCFA compared to the other two diets ($P < 0.001$, $P < 0.0001$ respectively). Plasma glycine (Figure 3.8) was increased by LCFA compared to MCFA and Control ($P < 0.001$), and serine (Figure 3.8) reached higher concentrations in plasma of LCFA fed pigs than MCFA fed pigs, with intermediate concentrations in pigs fed Control formula. Despite causing a reduction in plasma concentrations of other amino acids, MCFA doubled glutamate concentrations (Figure 3.8) compared to the other diets ($P < 0.0001$). Finally, plasma urea concentrations (Figure 3.8) decreased compared to Control by 35% with LCFA and by 47% with MCFA feeding ($P < 0.001$).

In the free amino acid pool of the longissimus dorsi muscle (Table 3.5), alanine concentration for MCFA pigs was less than that of LCFA or Control ($P < 0.05$). Glycine concentration was greater for pigs fed the LCFA formula compared with those in the Control or MCFA groups ($P < 0.01$). Methionine ($P < 0.01$) and serine ($P < 0.05$) concentrations were higher for LCFA fed pigs compared to MCFA, and intermediate for Control pigs.

In the liver (Table 3.6), valine was more concentrated for pigs fed MCFA than LCFA, and intermediate for pigs fed Control ($P < 0.05$). Leucine and isoleucine concentration followed a similar tendency to valine ($P = 0.07$, $P = 0.08$, respectively). In addition, hepatic methionine ($P < 0.05$) and phenylalanine ($P < 0.05$) concentrations were greater for MCFA fed pigs compared to Control, and were intermediate for LCFA fed pigs. Glutamine concentration tended to increase by 30% in MCFA fed pigs compared to LCFA and Control ($P = 0.07$).

Tissue gene and protein expression and substrate oxidation. Diet had no effect on translation initiation signaling in the longissimus dorsi (Figure 3.9). Furthermore, diet did not affect gene expression of BCAA catabolizing enzymes in the longissimus dorsi or liver (Figure 3.10).

Hepatic expression of acetyl-CoA carboxylase alpha (ACACA) and fatty acid synthase (FASN) mRNA (Figure 3.12) were greater for MCFA compared with Control and LCFA groups ($P < 0.01$). Gene expression of BCAA catabolizing genes were not affected by formula. However, oxidation of KIV (Figure 3.13) of pigs fed MCFA was 42% lower than those fed Control, and intermediate in those fed LCFA formula ($P < 0.05$). Finally, oxidation of KIC (Figure 3.13) was decreased in liver of pigs fed MCFA and LCFA compared to Control ($P < 0.001$).

Discussion

Plasma BCAA concentrations have been positively correlated with obesity and insulin resistance. However, a cause and effect relationship has not been established and it remains an active area of research. In a previous study, we reported that feeding diets enriched with BCAA did not alter adiposity or hepatic fat metabolism in neonatal pigs (Yonke et al., 2020). In the current study, we set out to determine whether metabolic disturbances related to fat accumulation in the liver precede elevated plasma BCAA.

It is evident from our plasma keto and amino acid concentration data that KIV disposal was reduced in pigs fed MCFA formula. Plasma KIV concentration was increased by MCFA, suggesting an early stage of BCAA dysfunction. Although plasma BCKA and BCAA concentrations are strongly positively correlated postprandially (Matthews et al., 1982), valine concentration was not elevated in MCFA fed pigs in the current experiment. Pre-prandial rate of leucine appearance equal to the other diets demonstrates that muscle protein breakdown did not contribute to plasma leucine concentration in pigs fed high fat diets. Therefore, plasma BCKA likely originated from decreased catabolism of BCKA as opposed to increased appearance of BCAA. However, it is unclear why MCFA decreased the rate of leucine appearance in the hours following a meal compared to the other diets.

Postprandial circulating leucine appears from the diet or from muscle protein breakdown. Muscle protein breakdown is unlikely contribute significantly to leucine appearance in MCFA fed pigs because they are in an anabolic state postprandially (El-Kadi et al., 2012). This is consistent with normal translation initiation signaling of MCFA fed pigs compared to other diets, and their normal pre-prandial rate of leucine appearance. However, it is possible that MCFA reduced protein degradation in response to a meal more than the other diets, which would be

consistent with improved insulin sensitivity suggested by improved glucose clearance in pigs fed MCFA. Delayed or decreased leucine absorption is the more likely cause of decreased appearance. The consistently delayed concentration peaks of most amino acids suggest delayed protein digestion in MCFA pigs. Significant exceptions include Asp being the only AA with a similar peak to the other diets, Gln hardly changing in response to diet, and Glu abnormally high in response to MCFA. These three amino acids are heavily used by the gastrointestinal tract, with low or negative net portal balance following a meal (Stoll et al., 1998). Plasma concentrations of these nonessential amino acids therefore largely result from endogenous synthesis. The gastrointestinal tract also uses a large portion of BCAA, with only about 60% of dietary and arterial BCAA reaching the portal vein following a meal (Stoll et al., 1998). Because high energy diets increase the mass of the gastrointestinal tract (King et al., 2004), and MCFA increase villus height in all regions of the intestine (Chwen et al., 2013), it is possible that MCFA increase amino acid removal by the gastrointestinal tract to support intestinal growth and secretions, resulting in decreased rate of leucine appearance and other decreases in amino acid concentration.

The large increase in plasma glutamate in response to MCFA could result from increased transaminase activity. Increased glutamate was also seen in obese human subjects in conjunction with elevated BCAA and decreased α-ketoglutarate (Newgard et al., 2009), which was proposed to result from increased cytosolic branched-chain amino acid transaminase (BCAT2) activity. The same results could originate from activity of other transaminases. Rats fed MCFA had increased hepatic alanine aminotransferase activity (Sugiyama et al., 2015). Another possibility is that glutamate was synthesized from α-ketoglutarate and amino acids in an attempt to remove

ammonia by glutamine synthesis (Holeček, 2021), which is supported by tissue glutamine concentrations in the current experiment.

Decreased BCKA oxidation was traced back to the liver of MCFA fed pigs, where both KIV and KIC oxidation were reduced in liver homogenate. While decreased BCKA oxidation in MCFA fed pigs could be viewed as a response to decreased amino acid availability, our data suggest that this is unlikely based on hepatic BCAA concentrations and branched-chain keto acid dehydrogenase E1 subunit alpha (BCKDHA) mRNA expression not differing between MCFA and Control fed pigs at 60 minutes following a meal. BCKA oxidation was not regulated by BCKDHA mRNA expression in this case. However, BCKDH is also regulated by phosphorylation, $NADH/NAD^+$ and acetyl-CoA concentration (Boyer & Odessey, 1990). Therefore, oxidation of large quantities of MCFA can potentially inhibit BCKDH activity. Although CO_2 production quickly plateaus with increased laurate concentration in rat hepatocytes, acid soluble metabolites continue to be produced linearly (Christensen et al., 1991). Acetyl-CoA accumulation from MCFA would inhibit BCKDH, slowing oxidation of all BCKA, including KIV which enters the TCA cycle as succinyl-CoA. Reducing input of glucogenic TCA intermediates from BCKA is postulated to contribute to anaplerotic stress, which further slows flux of carbon through the TCA cycle (Sunny et al., 2015).

The high fat diets in the current experiment produced a model of obesity by doubling body fat percentage compared to Control. However, only MCFA increased lipogenic gene expression and decreased oxidation of KIV. Among the reasons that MCFA are supplemented are that they are a highly metabolizable source of energy due to their direct transport to and high rate of oxidation in the liver (Heo et al., 2002; Odle, 1991) which reduces plasma lipid profiles (Y. Liu et al., 2017; Zhang et al., 2018). Despite the potentially beneficial properties of MCFA,

increased ACACA and FASN expression demonstrate that livers of MCFA fed pigs store more energy in the liver than pigs fed the other diets. Others have reported increased lipogenic gene expression in the liver of pigs fed coconut oil (Kellner et al., 2017), and in the livers of rodents fed low (Tucci et al., 2010) and high (Ronis et al., 2013) amounts of MCT. Hepatic de novo lipogenesis is particularly abnormal in swine because that function is primarily designated to adipose tissue. When MCFA are fed in amounts exceeding the liver's ability to completely oxidize them, MCFA are used to synthesize LCFA. Rat hepatocytes rapidly converted the majority added laurate to palmitate in cultures (Rioux et al., 2003). Our data suggest that formulas rich in dietary fat altered hepatic fat metabolism and that these changes precede dysfunctional BCAA catabolism.

A milk replacer formula high in MCFA or LCFA was fed in the current study to determine whether energy supplementation would have an effect on liver function and as a result contribute to BCAA dysregulation. The liver of neonatal pigs as a model for chronic conditions associated with liver lipid dysfunction including obesity and insulin resistance. Insulin resistance becomes a confounding factor in studying the relationship between obesity and BCAA metabolism because it is a common comorbidity of obesity, and insulin resistance affects BCAA metabolism independently of obesity (Tan et al., 2017). Increased plasma BCAA can also affect insulin sensitivity through chronic mTOR activation (Newgard et al., 2009; Zakaria et al., 2021). Our MCFA model circumvents the insulin resistance factor because MCFA improved glucose homeostasis in our pigs as they did in rodent models (Geng et al., 2016; E.-J. Lee et al., 2018; H. Sun et al., 2013; M.-E. Wang et al., 2017; Wein et al., 2009). Improved glucose clearance in response to MCFA has been attributed to improved insulin sensitivity in skeletal muscle in mice which concurrently developed hepatic insulin resistance and steatosis (Turner et al., 2009).

In conclusion, we set out to determine whether high fat diets of LCFA and MCFA precede BCAA metabolic dysregulation in neonatal pigs. Our data demonstrate that MCFA dysregulate hepatic fat metabolism and decrease BCKA oxidation. Therefore, decreased oxidation of BCKA is likely causal to increased plasma BCAA concentrations associated with high fat diets and obesity long term.

Table 3.1. Composition of experimental diets (%, as fed).

Item	Dietary Treatment		
	Control	LCFA	MCFA
Ingredient			
Whey protein isolate* (90% CP)	5.64	5.63	5.64
Lactose*	0.47	0	1.01
Fat Pak 80†	5.39	10.07	0
Virgin Coconut Oil	0	0	8.76
Mineral premix†‡	0.90	0.90	0.90
Dicalcium phosphate	0.56	0.51	0.62
Vitamin premix†§	0.20	0.20	0.20
Xanthan gum†	0.10	0.10	0.10
Calcium carbonate	0.16	0.19	0.14
Casein Powder	0.22	0	0.46
Water	86.36	82.40	82.18
Calculated nutrient composition			
ME, *kcal/kg*	656	984	984
Crude protein	5.25	5.25	5.25
Crude fat	4.43	8.17	8.78
Lactose	0.97	0.97	0.97
Calcium	0.23	0.23	0.23
Phosphorus	0.14	0.14	0.14
Amino acids			
Arginine	0.15	0.15	0.15
Histidine	0.10	0.10	0.10
Isoleucine	0.29	0.29	0.29
Leucine	0.55	0.55	0.55
Lysine	0.48	0.48	0.48
Methionine	0.12	0.12	0.12
Phenylalanine	0.17	0.17	0.17
Threonine	0.35	0.35	0.35
Tryptophan	0.086	0.086	0.087
Valine	0.30	0.30	0.30

Control = low protein, low energy; LCFA = low protein, high energy from animal fat blend; MCFA = low protein, high energy from virgin coconut oil.
*Milk Specialties, Eden Prairie, MN.
†Dyets Inc., Bethlehem, PA.
‡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.
§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.

Table 3.2. Gene specific primers used for RT-qPCR

Gene	Direction	Primer Sequence	
PPIA	Forward	5’ – CGTCTTCTTCGACATCGCCG – 3’	NM_214353.1
	Reverse	5’ – GAAGTCACCACCCTGGCACATAAAT – 3’	
H3F3A	Forward	5’ – GGTAAGTAAGGAGGTCTCTATACCA – 3’	NM_213930.1
	Reverse	5’ – TAATTTCACGGAGTGCCACG – 3’	
B2M	Forward	5’ – TTCAGGTTTACTCACGCCAC – 3’	NM_213978.1
	Reverse	5’ – GAACTCAGTGTGGACCAGAAGG -3’	
TBP	Forward	5’ – TTGTGCCACAGCTGCAAAAT – 3’	XM_021085493.1
	Reverse	5’ – AATATCAGTGCGGTGGTCCG – 3’	
BCAT2	Forward	5’ – CCT CTT CCG ACC TTG GCT CA – 3’	XM_013998608.2
	Reverse	5’ – ACC GGG CAG AGG ATG ACG TA – 3’	
BCKDHA	Forward	5’ – TGG ACC GCA TCC TCT ACG AG – 3’	NM_001123083.1
	Reverse	5’ – AGG TCG CTC ACG TTG CTG TA – 3’	
ACACA	Forward	5’ – GGCCATCAAGGACTTCAACC – 3’	NM_001114269.1
	Reverse	5’ – ACGATGTAAGCGCCGAACTT – 3’	
FASN	Forward	5’ – TCAACCCCAACGGTCTGTTC – 3’	NM_001099930.1
	Reverse	5’ – CGGGCTGACGTCGAACTTAT – 3’	
PPARGC1A	Forward	5’ – GAGTCTGAAAGGGCCAAGCA – 3’	NM_213963.2
	Reverse	5’ – CAGTTCTGTCCGTGTTGTGTCA – 3’	
TFAM	Forward	5’ – GGTCCATCACAGGTAAAGCTGAA – 3’	NM_001130211.1
	Reverse	5’ – CGTTTCGCCCAACTTCAACC – 3’	

Table 3.3. Growth performance of pigs fed either a low energy diet (Control, n=5) or high energy diet from long-chain (LCFA, n=6) or medium-chain fatty acids (MCFA, n=6) over 22 days.*

	Treatment				
	Control	LCFA	MCFA	SEM	Significance (P-value)
Average daily gain, kg	0.15	0.19	0.15	0.016	0.30
Total gain, kg	3.4	4.1	3.4	0.35	0.30
Total feed intake, L	20	21	19	1.6	0.72
Gain to feed ratio	0.16[b]	0.20[a]	0.17[ab]	0.0086	0.02
Fat gain, g	74[b]	370[a]	320[a]	37	0.0002
Lean gain, kg	3.2	3.5	2.9	0.30	0.40
Energy accretion, Mcal	4.3[b]	7.6[a]	6.5[ab]	0.0021	0.007
Protein accretion, g	630	710	580	60	0.39
Energy efficiency, %	32	37	33	1.9	0.13
Protein efficiency, %	58	66	56	3.1	0.09

*Results are least-square means. Means in a row without a common letter differ, $P \leq 0.05$. LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid; SEM, standard error of the mean. Gain to feed ratio was calculated as kilogram body weight gained per liter of formula consumed.

Table 3.4. Organ weights and hepatic fat content of pigs fed either a low energy diet (Control, n=5) or high energy diet from long-chain (LCFA, n=6) or medium-chain fatty acids (MCFA, n=6) over 22 days.*

	Treatment				
	Control	LCFA	MCFA	SEM	Significance (P-value)
Tissue weight, *g*					
Liver	220[b]	310[a]	310[a]	26	0.05
Longissimus	100	99	89	7.6	0.48
Soleus	4.5	4.8	3.9	0.30	0.12
Heart	42	45	46	4.1	0.73
Kidney	20	24	26	2.0	0.22
Tissue weight, *% body*.					
Liver	3.6[b]	4.7[a]	5.2[a]	0.24	0.001
Longissimus	1.6	1.5	1.5	0.062	0.25
Soleus	0.074	0.074	0.066	0.0028	0.11
Heart	0.69	0.67	0.78	0.036	0.10
Kidney	0.33[b]	0.36[b]	0.43[a]	0.018	0.001
Liver fat, *% liver*	16	26	17	3.5	0.14

*Results are least-square means. Means in a row without a common letter differ, $P \leq 0.05$. LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid; SEM, standard error of the mean.

Table 3.5. Free amino acid concentrations in longissimus dorsi muscle of pigs fed either a low energy diet (Control, n=5) or high energy diet from long-chain (LCFA, n=6) or medium-chain fatty acids (MCFA, n=6) over 22 days.*

Amino Acid, *μmol·g⁻¹ tissue*	Treatment			SEM	Significance (P-value)
	Control	LCFA	MCFA		
Essential					
Histidine	0.0631	0.0602	0.0781	0.011	0.45
Isoleucine	0.251	0.273	0.303	0.028	0.43
Leucine	0.390	0.411	0.440	0.042	0.69
Lysine	0.743	0.861	0.819	0.13	0.83
Methionine	0.0688[ab]	0.0944[a]	0.0505[b]	0.0082	0.006
Phenylalanine	0.0915	0.112	0.115	0.010	0.19
Threonine	2.81	3.70	2.34	0.39	0.07
Valine	0.370	0.373	0.440	0.060	0.65
Nonessential					
Alanine	1.79[a]	1.69[ab]	1.04[b]	0.19	0.03
Aspartate	1.230	0.870	1.210	0.17	0.26
Glutamate	4.86	4.03	4.28	0.88	0.79
Glutamine	11.9[ab]	10.2[b]	15.6[a]	1.3	0.02
Glycine	3.70[b]	5.62[a]	3.35[b]	0.41	0.004
Proline	1.72	1.72	1.36	0.18	0.30
Serine	0.760[ab]	1.040[a]	0.579[b]	0.12	0.04
Tyrosine	9.42	7.55	10.30	2.89	0.06

*Results are least-square means. Means in a row without a common letter differ, $P \leq 0.05$. LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid; SEM, standard error of the mean.

Table 3.6. Free amino acid concentrations in liver of pigs fed either a low energy diet (Control, n=5) or high energy diet from long-chain (LCFA, n=6) or medium-chain fatty acids (MCFA, n=6) over 22 days.*

Amino Acid, *μmol·g⁻¹ tissue*	Treatment			SEM	Significance (P-value)
	Control	LCFA	MCFA		
Essential					
Histidine	0.421	0.292	0.354	0.035	0.07
Isoleucine	0.428	0.390	0.472	0.025	0.08
Leucine	0.774	0.749	0.891	0.049	0.07
Lysine	0.779	1.040	0.721	0.14	0.24
Methionine	0.135[b]	0.162[ab]	0.206[a]	0.017	0.02
Phenylalanine	0.217[b]	0.231[ab]	0.293[a]	0.021	0.04
Threonine	1.28	1.49	1.57	0.25	0.73
Valine	0.614[ab]	0.530[b]	0.661[a]	0.031	0.04
Nonessential					
Alanine	2.13	2.16	2.18	0.26	0.99
Aspartate	4.96	7.30	6.20	0.86	0.20
Glutamate	5.39	5.37	6.86	0.90	0.42
Glutamine	5.58	6.38	8.30	0.77	0.07
Glycine	3.21	3.74	3.19	0.33	0.41
Proline	0.793	0.668	0.831	0.091	0.37
Serine	1.01	1.24	1.03	0.10	0.24
Tyrosine	4.32	3.81	3.28	0.96	0.52

*Results are least-square means. Means in a row without a common letter differ, $P \leq 0.05$. LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid; SEM, standard error of the mean.

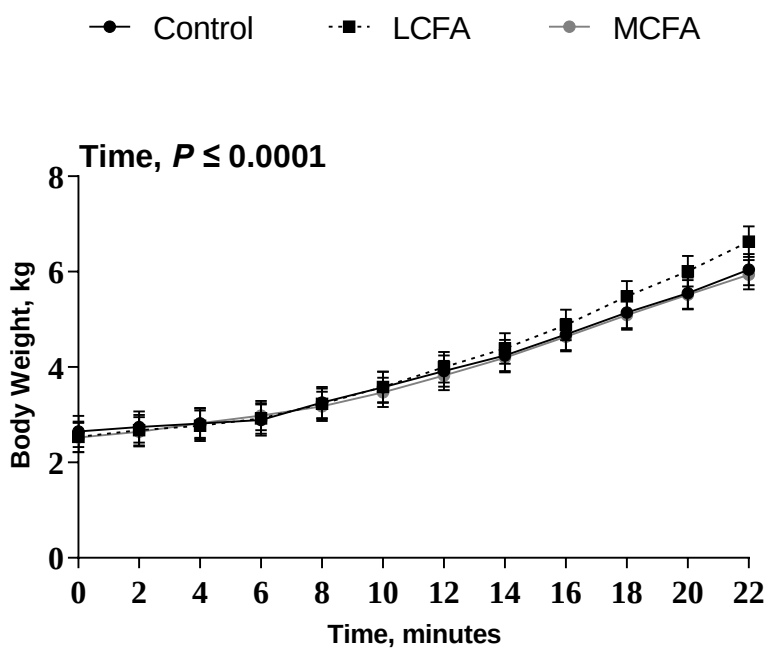


Figure 3.1. Cumulative body weight of pigs fed either a low energy diet (Control, n=5) or high energy diet from long-chain (LCFA, n=6) or medium-chain fatty acids (MCFA, n=6) over 22 d. Results are means ± SEMs. LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid.

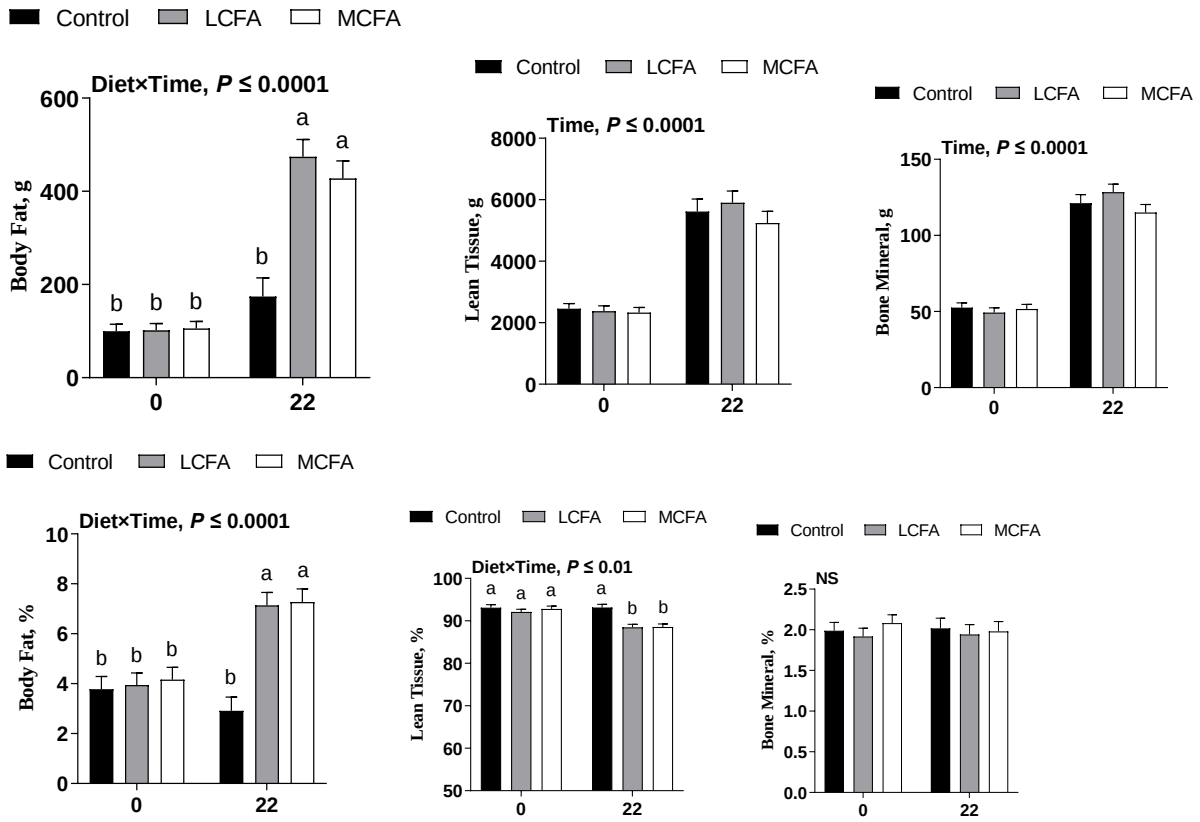


Figure 3.2. Body composition of pigs before beginning experimental diets and after fed a low energy diet (Control, n=5), or high energy diet from long-chain (LCFA, n=6) or medium-chain fatty acids (MCFA, n=6) for 22 d. Results are means ± SEMs. For the main effect or interaction shown, means without a common letter differ, $P \leq 0.05$. LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid.

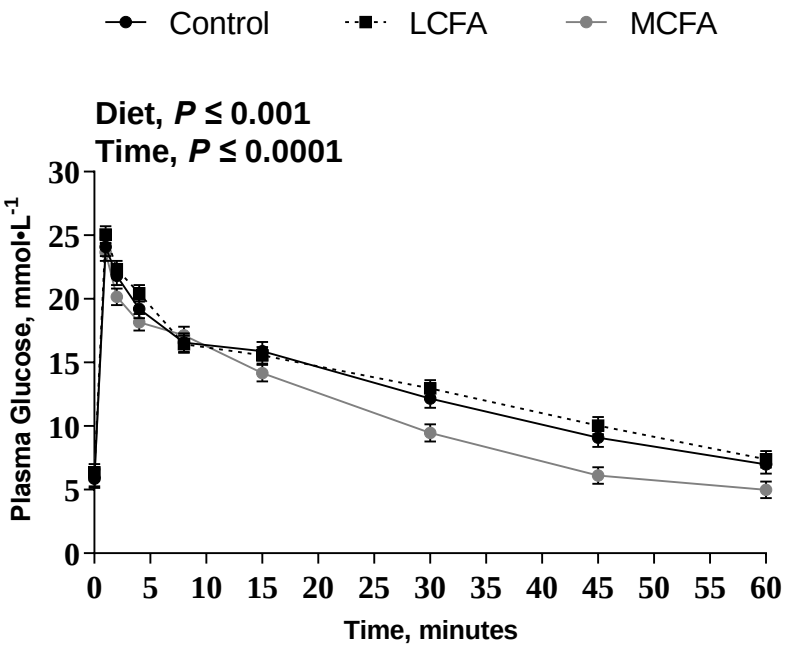


Figure 3.3. Plasma glucose concentration during an intravenous glucose tolerance test of pigs fed a low energy diet (Control, n=5), or high energy diet from long-chain (LCFA, n=6) or medium-chain fatty acids (MCFA, n=6) for 18 days. Results are means ± SEMs. LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid.

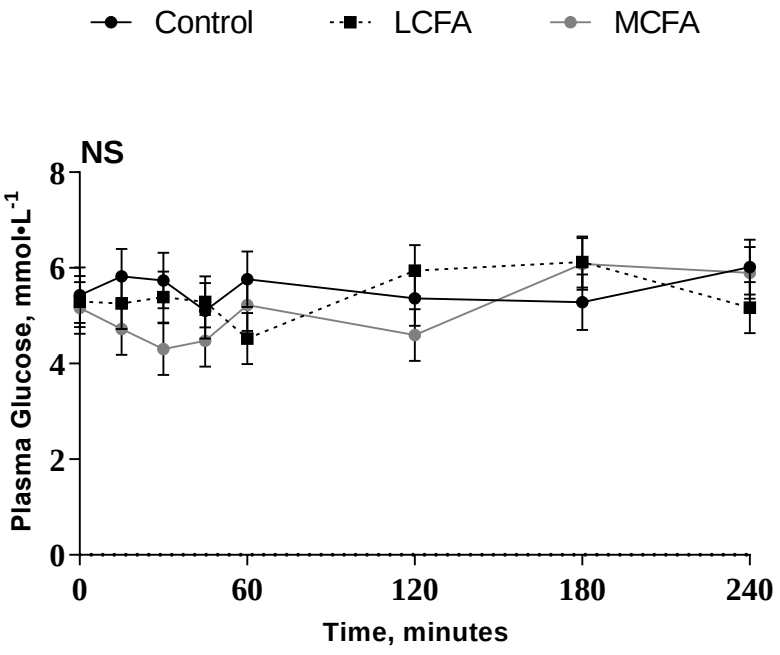


Figure 3.4. Postprandial plasma glucose concentration of pigs fed a low energy diet (Control, n=5), or high energy diet from long-chain (LCFA, n=6) or medium-chain fatty acids (MCFA, n=6) for 22 days. Results are means ± SEMs. LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid.

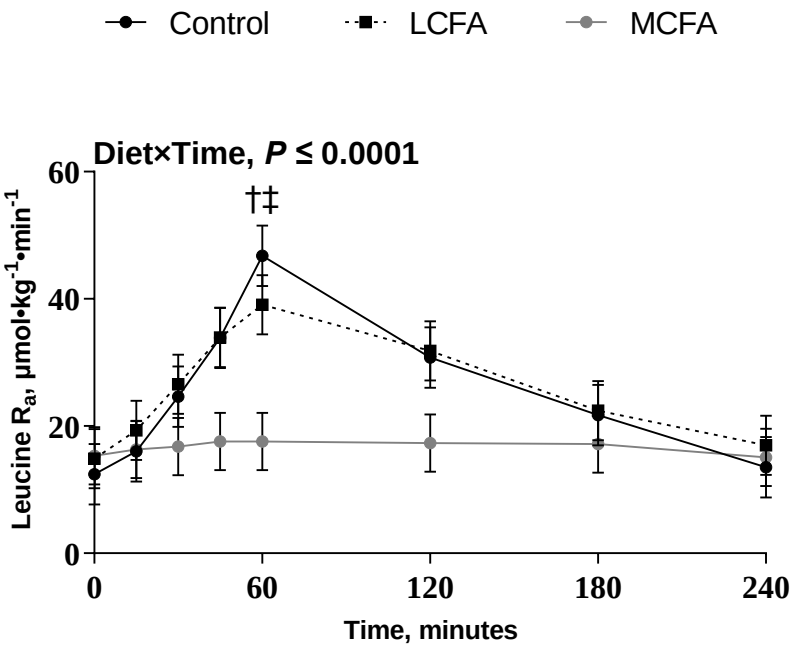
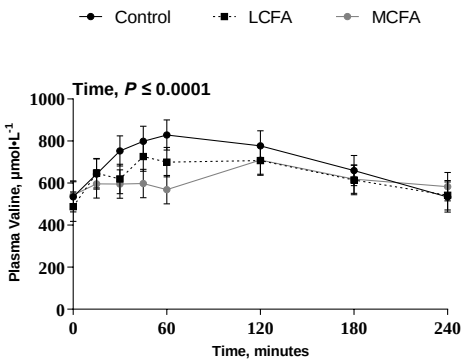
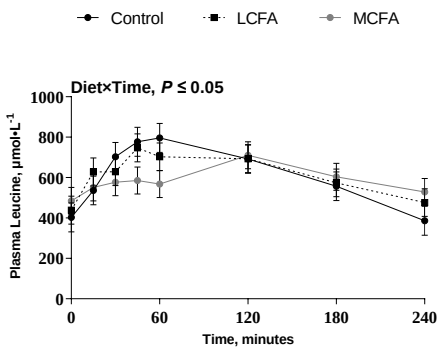
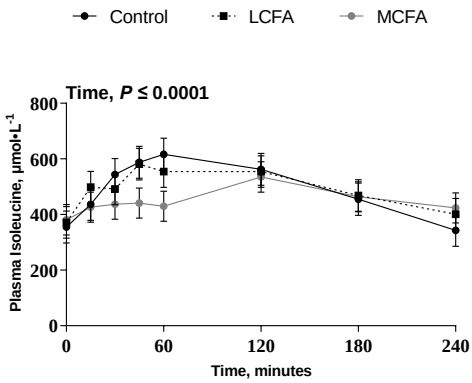
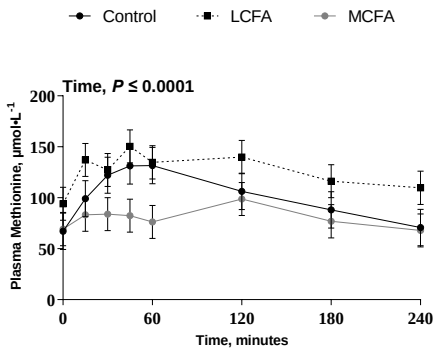


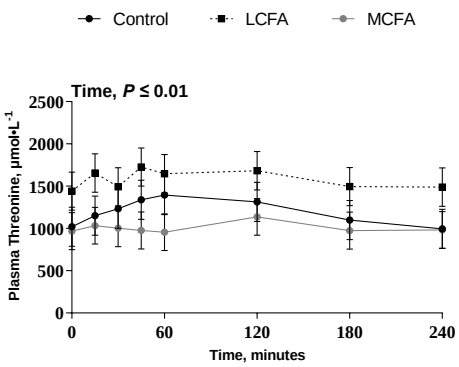
Figure 3.5. Whole body rate of plasma leucine appearance following a meal in pigs fed a low energy diet (Control, n=5), or high energy diet from long-chain (LCFA, n=6) or medium-chain fatty acids (MCFA, n=6) for 22 days. Results are means ± SEMs. Differences between dietary treatments within each timepoint according to Tukey–Kramer multiple comparison test ($P \leq 0.05$) are indicated as, "*", Control vs. LCFA; "†", Control vs MCFA; "‡"' LCFA vs. MCFA. LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid.

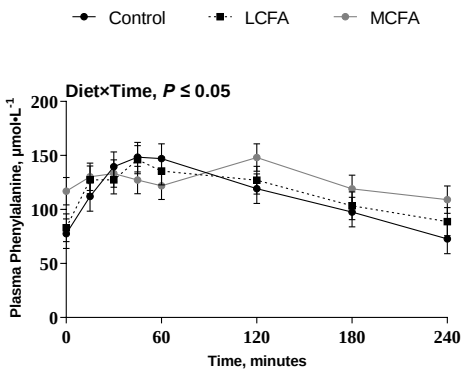

Control
LCFA
MCFA
Time, P ≤ 0.0001
Plasma Valine, µmol•L-1
Time, minutes


Control
LCFA
MCFA
Diet×Time, P ≤ 0.05
Plasma Leucine, µmol•L-1
Time, minutes


Control
LCFA
MCFA
Time, P ≤ 0.0001
Plasma Isoleucine, µmol•L-1
Time, minutes


Control
LCFA
MCFA
Time, P ≤ 0.0001
Plasma Methionine, µmol•L-1
Time, minutes


Control
LCFA
MCFA
Time, P ≤ 0.01
Plasma Threonine, µmol•L-1
Time, minutes


Control
LCFA
MCFA
Diet×Time, P ≤ 0.05
Plasma Phenylalanine, µmol•L-1
Time, minutes

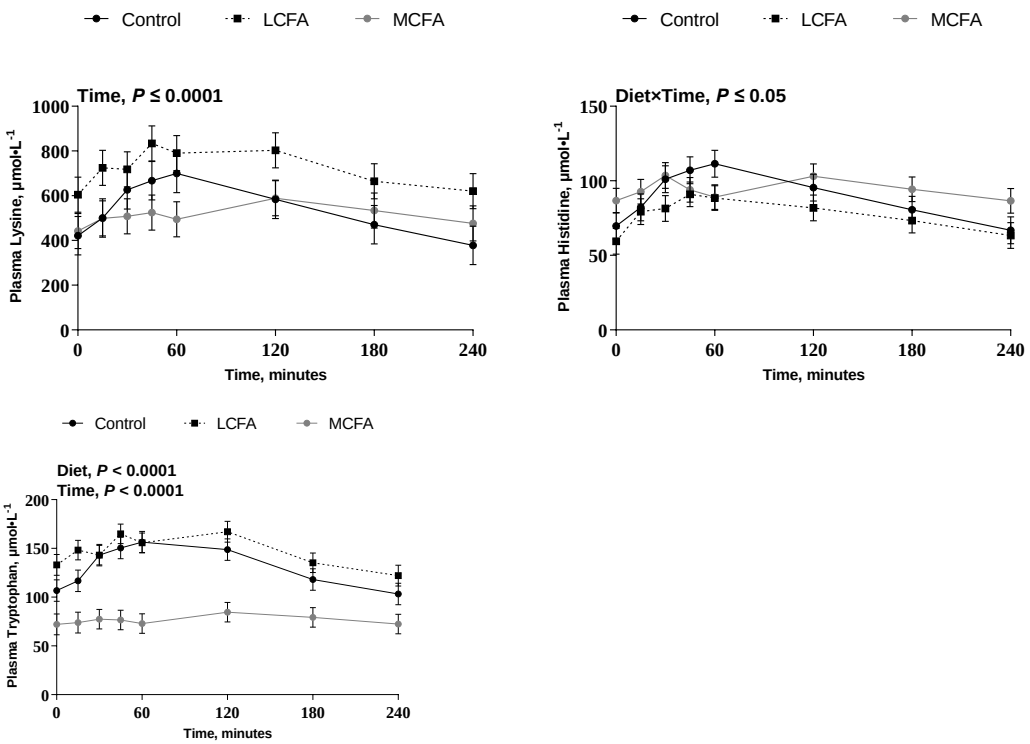


Figure 3.6. Plasma essential amino acid concentrations following a meal in in pigs fed a low energy diet (Control, n=5), or high energy diet from long-chain (LCFA, n=6) or medium-chain fatty acids (MCFA, n=6) for 22 days. Results are means ± SEMs. LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid.

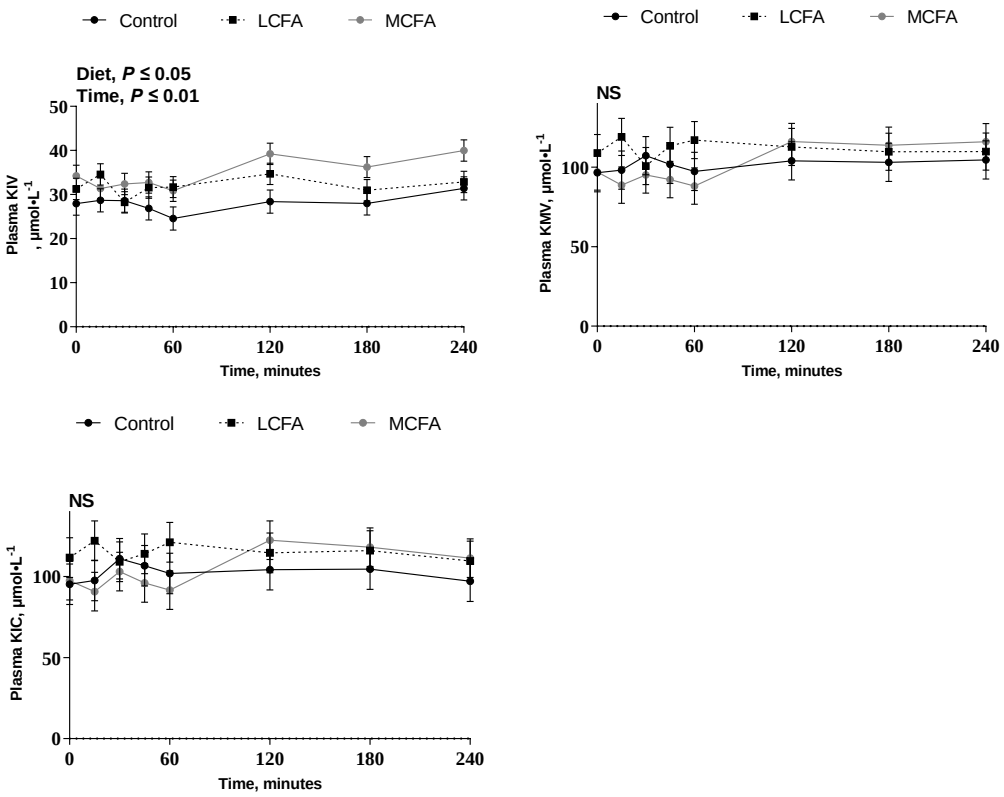
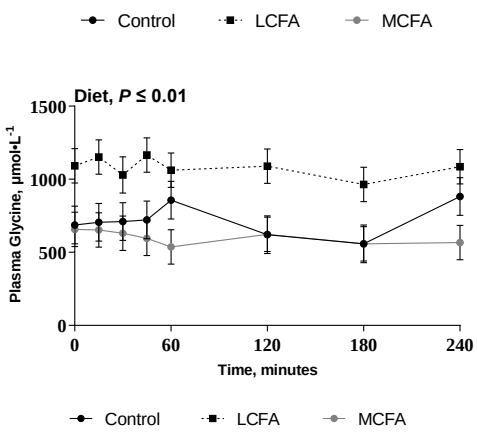
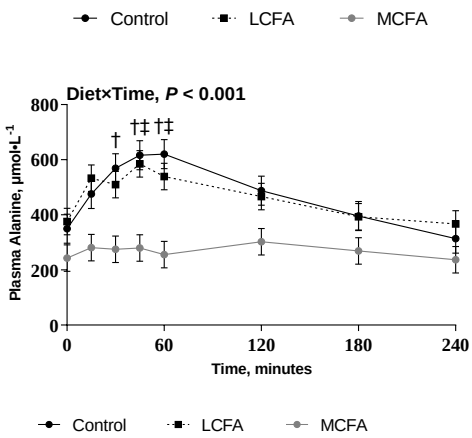
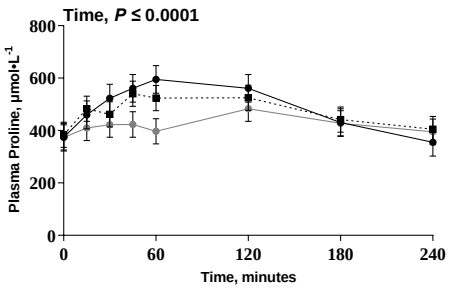
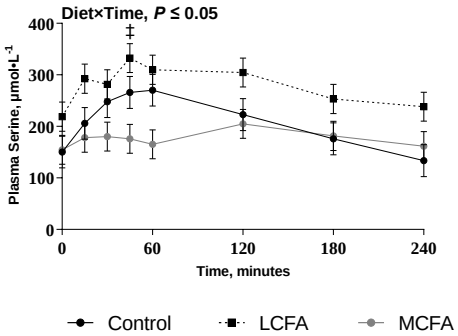


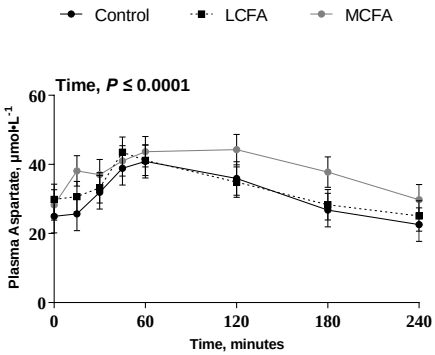
Figure 3.7. Plasma branched-chain α-keto acid concentrations following a meal in in pigs fed a low energy diet (Control, n=5), or high energy diet from long-chain (LCFA, n=6) or medium-chain fatty acids (MCFA, n=6) for 22 days. Results are means ± SEMs. LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid; KIV, α-ketoisovaleric acid; KMV, α-ketomethylvaleric acid; KIC, α-ketoisocaproic acid.

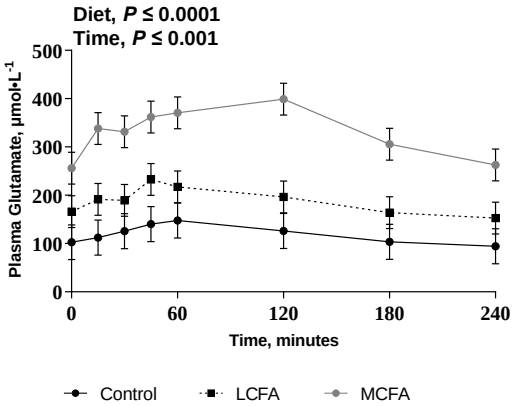

Control
LCFA
MCFA
Diet, P ≤ 0.01
Plasma Glycine, µmol•L-1
Time, minutes

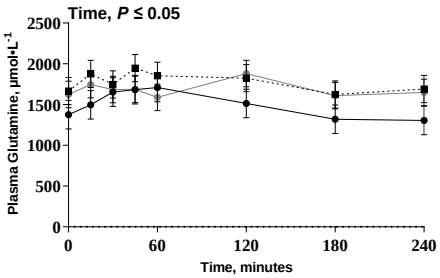

Control
LCFA
MCFA
Diet×Time, P < 0.001
Plasma Alanine, µmol•L-1
Time, minutes


Control
LCFA
MCFA
Time, P ≤ 0.0001
Plasma Proline, µmol•L-1
Time, minutes


Diet×Time, P ≤ 0.05
Plasma Serine, µmol•L-1
Time, minutes
Control
LCFA
MCFA


Control
LCFA
MCFA
Time, P ≤ 0.0001
Plasma Aspartate, µmol•L-1
Time, minutes


Diet, P ≤ 0.0001
Time, P ≤ 0.001
Plasma Glutamate, µmol•L-1
Time, minutes
Control
LCFA
MCFA


Control
LCFA
MCFA
Time, P ≤ 0.05
Plasma Glutamine, µmol•L-1
Time, minutes

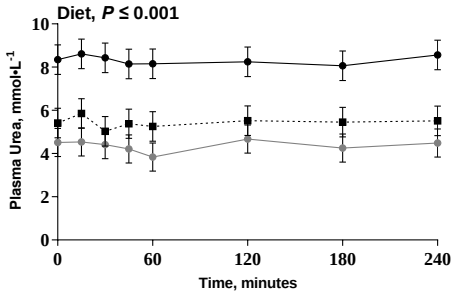
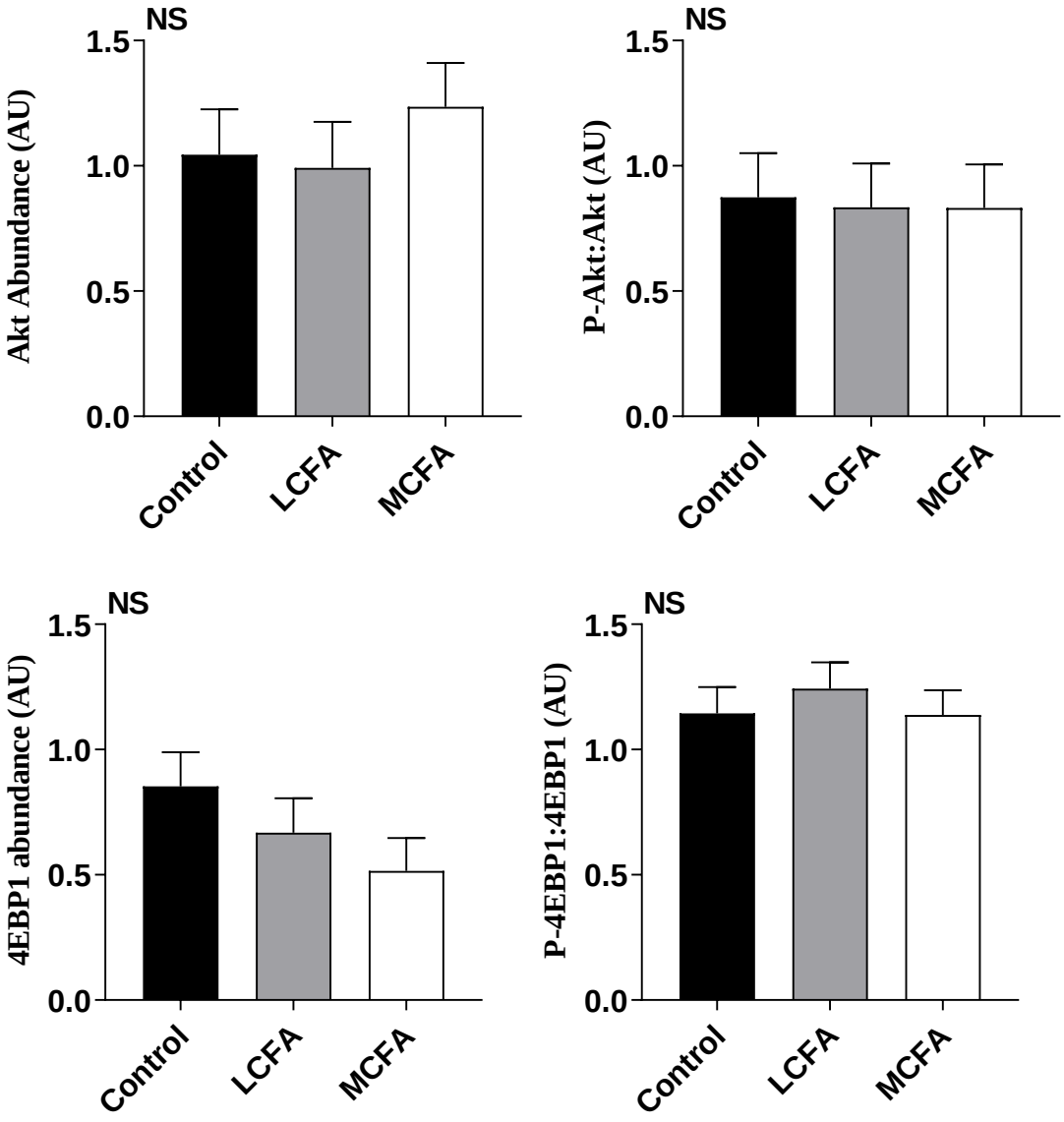

Diet, P ≤ 0.001
Plasma Urea, mmol•L-1
Time, minutes

Figure 3.8. Plasma non-essential amino acid and urea concentrations following a meal in pigs fed a low energy diet (Control, n=5), or high energy diet from long-chain (LCFA, n=6) or medium-chain fatty acids (MCFA, n=6) for 22 days. Results are means ± SEMs. Differences between dietary treatments within each timepoint according to Tukey–Kramer multiple comparison test ($P \leq 0.05$) are indicated as, "*", Control vs. LCFA; "†", Control vs MCFA; "‡"' LCFA vs. MCFA. LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid.


NS
1.5
1.0
0.5
0.0
Akt Abundance (AU)
Control
LCFA
MCFA
NS
1.5
1.0
0.5
0.0
P-Akt:Akt (AU)
Control
LCFA
MCFA
NS
1.5
1.0
0.5
0.0
4EBP1 abundance (AU)
Control
LCFA
MCFA
NS
1.5
1.0
0.5
0.0
P-4EBP1:4EBP1 (AU)
Control
LCFA
MCFA

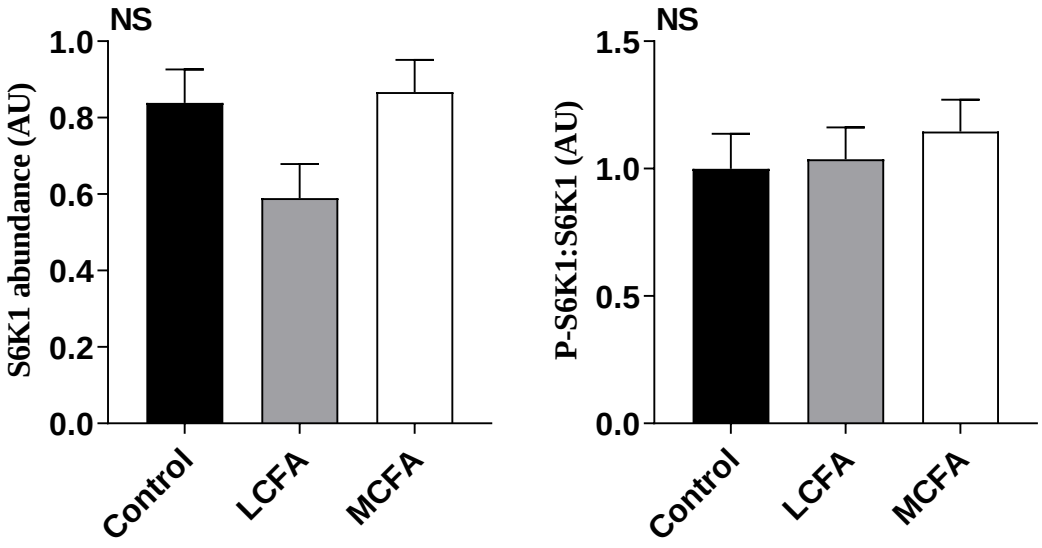


Figure 3.9. Relative protein abundance of translation initiation signaling proteins in the longissimus dorsi of pigs fed a low energy diet (Control, n=5), or high energy diet from long-chain (LCFA, n=6) or medium-chain fatty acids (MCFA, n=6) for 22 days. Results are means ± SEMs. LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid.

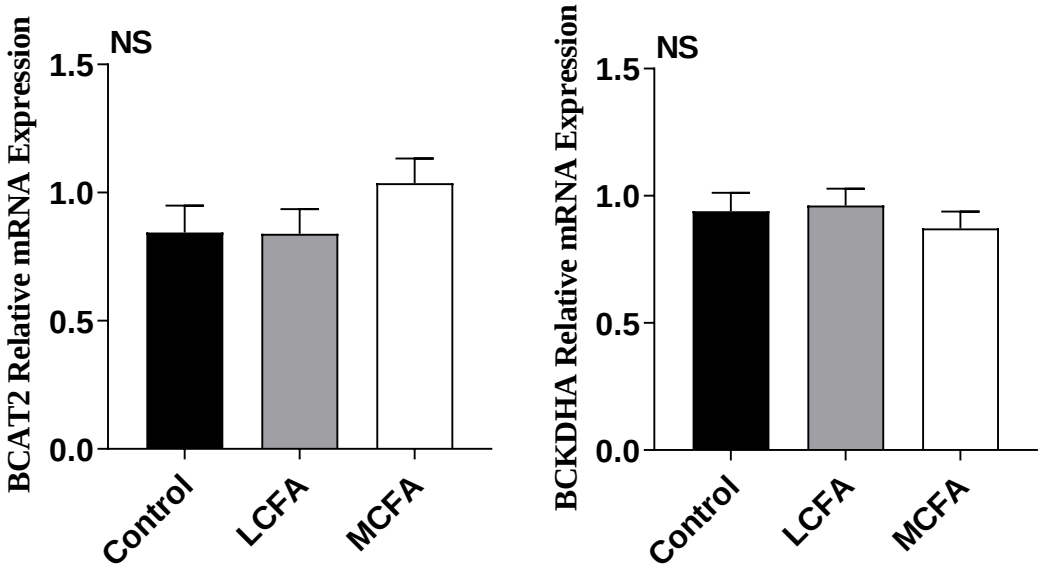


Figure 3.10. Relative gene expression of branched-chain amino acid metabolizing enzymes in the longissimus dorsi of pigs fed a low energy diet (Control, n=5), or high energy diet from long-chain (LCFA, n=6) or medium-chain fatty acids (MCFA, n=6) for 22 days. Results are means ± SEMs. LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid; BCAT2, branched-chain amino acid transaminase, mitochondrial; BCKDHA, branched-chain keto acid dehydrogenase E1 subunit alpha.

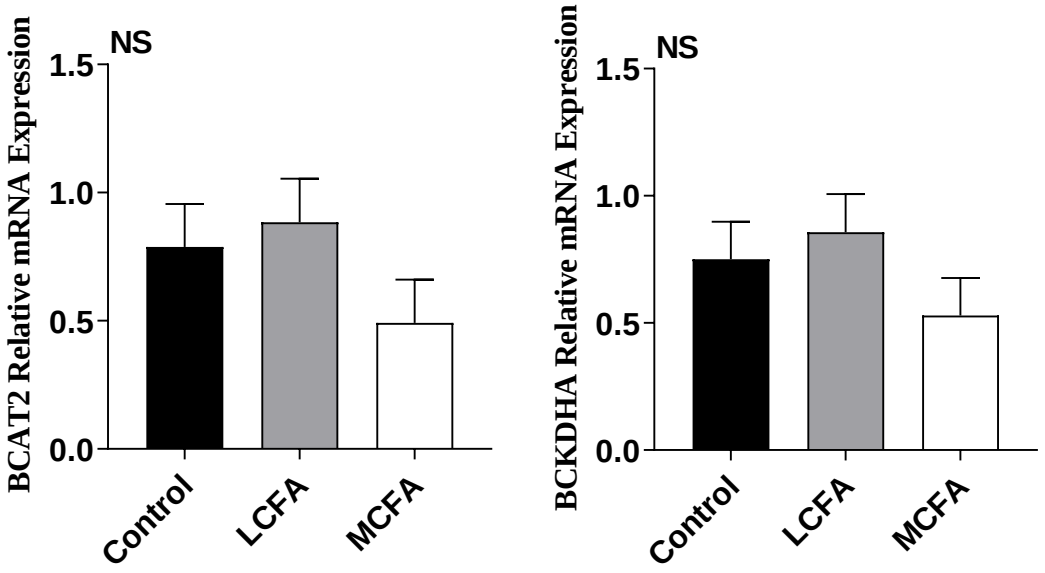


Figure 3.11. Relative expression of branched-chain amino acid metabolizing genes in the liver of pigs fed a low energy diet (Control, n=5), or high energy diet from long-chain (LCFA, n=6) or medium-chain fatty acids (MCFA, n=6) for 22 days. Results are means ± SEMs. LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid; BCAT2, branched-chain amino acid transaminase, mitochondrial; BCKDHA, branched-chain keto acid dehydrogenase E1 subunit alpha.

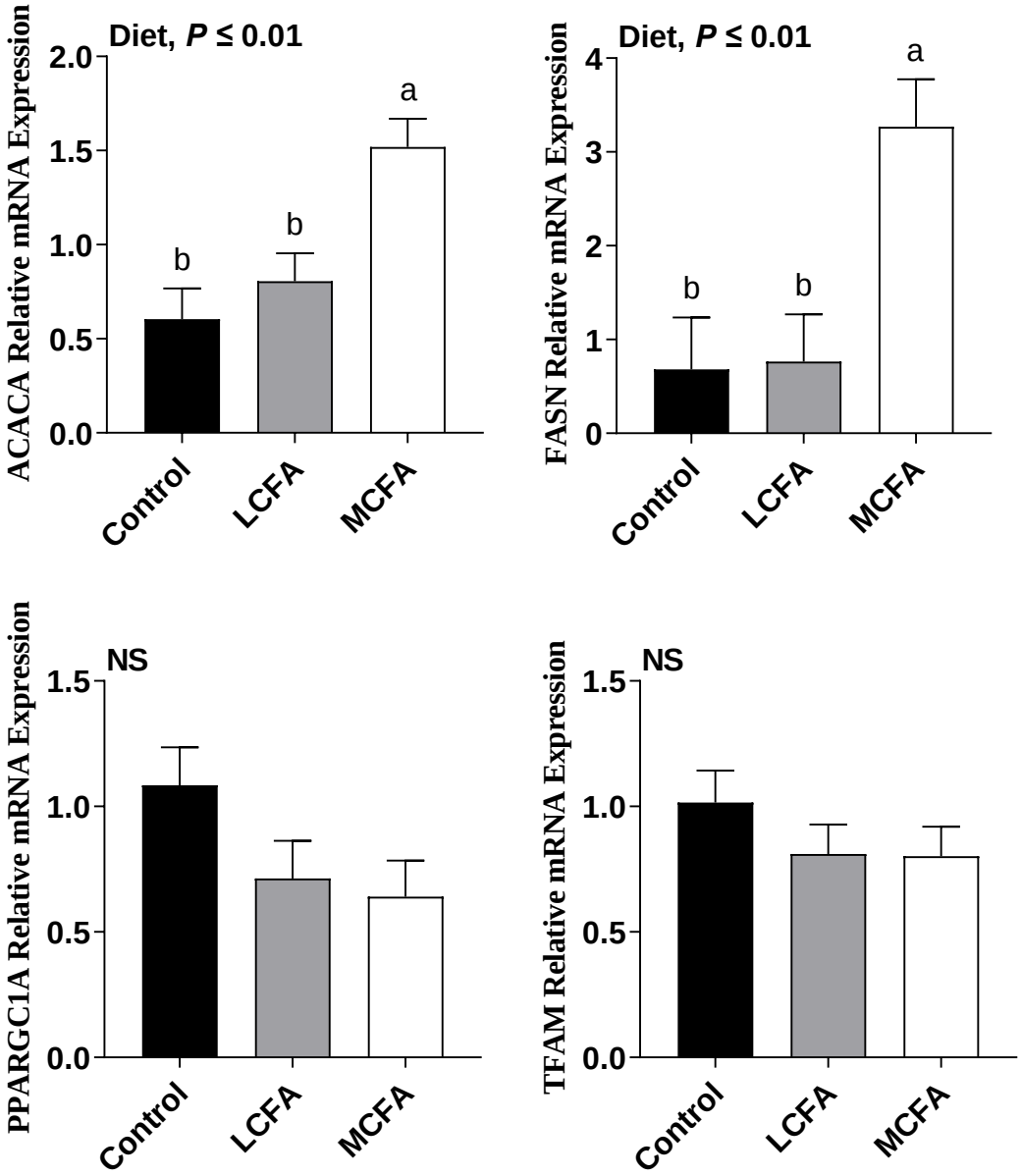


Figure 3.12. Relative expression of lipogenic and mitochondria biogenic genes in the liver of pigs fed a low energy diet (Control, n=5), or high energy diet from long-chain (LCFA, n=6) or medium-chain fatty acids (MCFA, n=6) for 22 days. Results are means ± SEMs. For the main effect shown, means without a common letter differ, $P \leq 0.05$. LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid; ACACA, acetyl-CoA carboxylase alpha; FASN, fatty acid synthase; PPARGC1A, peroxisome proliferator activated receptor gamma coactivator 1 alpha; TFAM, transcription factor A, mitochondrial.

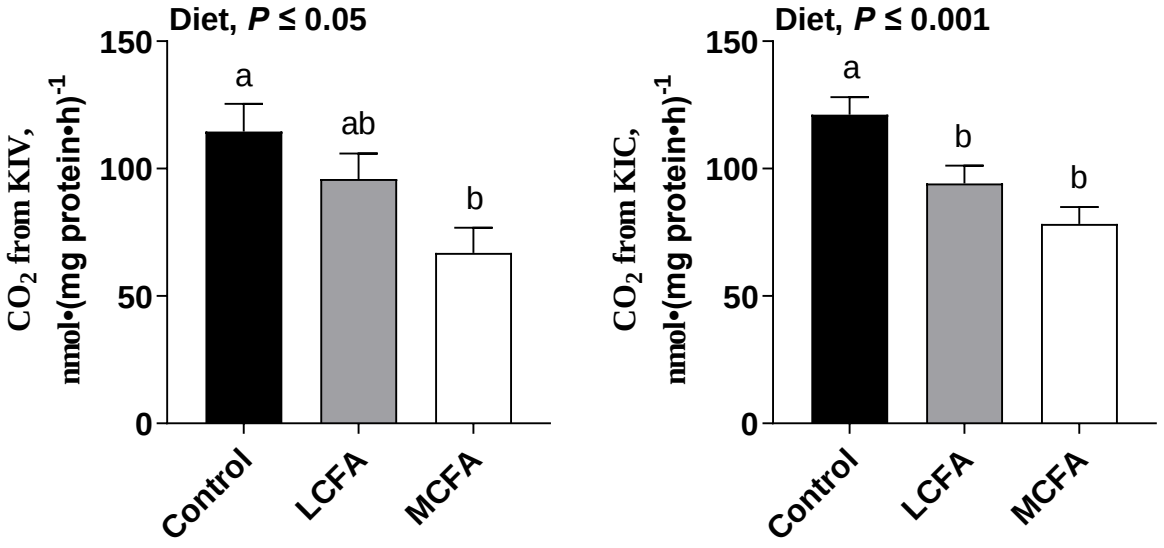


Figure 3.13. Oxidation of branched-chain alpha-keto acids to CO2 in liver homogenate of pigs fed a low energy diet (Control, n=5), or high energy diet from long-chain (LCFA, n=6) or medium-chain fatty acids (MCFA, n=6) for 22 days. Results are means ± SEMs. For the main effect shown, means without a common letter differ, $P \leq 0.05$. LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid; KIV, α-ketoisovaleric acid; KIC, α-ketoisocaproic acid.

Chapter 4. Summary

Milk replacement formula is often fed to infants who are unable to consume breast milk. LBWT infants, in particular, are already prone to decreased muscle growth which results in metabolic health issues later in life. It is important to develop methods of formula feeding which maximize lean growth in order to recover muscle mass as early as possible. In this research, BCAA were investigated as a means to stimulate protein synthesis and lean accretion. However, BCAA have previously also been positively correlated with obesity and metabolic disease. Because LBWT infants are already at increased risk of these health issues, this research also aimed to demonstrate that BCAA do not cause obesity or metabolic dysfunction. The overall hypothesis of this research was that metabolic dysfunction from high fat diets precedes dysfunctional BCAA catabolism.

Animals detect leucine as an indicator that amino acids are available for muscle protein synthesis. However, the protein synthesis initiating function of leucine has yet to be proven effective for increasing muscle mass when fed to LBWT infants long term. In the first study, by feeding BCAA supplemented diets for 4 weeks, we provided LBWT and NBWT pigs sufficient nutrition and ample time to grow. At the conclusion of the feeding period, Sestrin2 expression in skeletal muscle of LBWT pigs was less than half of that expressed in NBWT pigs. Unless it is bound to leucine, Sestrin2 inhibits mTOR, so less Sestrin2 means less leucine would theoretically be required to activate mTOR. Additionally, protein synthetic capacity was increased by BCAA. Yet, BCAA supplementation had no effect on mTOR activation or lean growth of LBWT or NBWT pigs. Lack of anabolic signal induction suggests that the effect of leucine on protein synthesis decreases over an extended period of supplementation.

In addition to demonstrating that BCAA supplementation did not increase lean growth in neonatal pigs, we also showed that 4 weeks of high BCAA consumption did not result in adiposity or metabolic dysfunction. Neonatal pigs grow extremely rapidly, so differences in adiposity develop quickly, as demonstrated when high fat diets were fed in the second experiment. Additionally, the results suggest that BCAA supplementation is not likely to contribute to the prevalence of neonatal NAFLD, based on very low hepatic fat accumulation in all groups of pigs, and normal expression genes involved in fat transport, lipogenesis, and mitochondrial biogenesis. Based on this research, high BCAA consumption is safe for neonatal pigs, and is not the cause of the positive correlation between plasma BCAA concentration and obesity or metabolic disease.

Having concluded that elevating plasma BCAA concentrations does not precede adiposity and metabolic dysfunction in neonatal pigs, the second experiment aimed to demonstrate that high fat diets lead to increased plasma BCAA concentrations. As expected, high fat diets of LCFA and MCFA increased adiposity compared to the low energy control diet. Both high fat diets doubled body fat percentage of pigs compared to Control. Interestingly, MCFA caused more metabolic disturbances than LCFA. Coconut oil and MCFA have a good reputation for being highly metabolizable. However, 87% of energy in the diet from coconut oil appeared to overwhelm the liver's ability to oxidize it. Compared to the Control and LCFA formulas, MCFA increased lipogenic gene expression more than twofold in the liver, and impaired BCKA oxidation. Plasma KIV concentration also increased in MCFA fed pigs. The effect of MCFA on the terminal step of BCAA catabolism suggests that high fat diets can impair oxidative disposal of BCAA. Eventually, when fewer BCKA are oxidized to CO_2, more BCKA must either be reaminated back to BCAA, increasing plasma concentrations, or more KIC and

KMV carbon must be converted to fatty acids or ketone bodies, and more KIV and KMV carbon must be converted to glucose.

Overall, these studies concluded that high fat diets cause impairments in BCAA catabolism which are likely to precede elevated plasma BCAA concentrations. BCAA supplementation does not increase muscle growth in neonatal pigs, but there is also no indication that it causes obesity or dysfunctional fat metabolism. To build upon this research, it would be informative to feed a high MCFA diet for a longer duration in order to determine whether plasma BCAA concentrations increase, and whether NAFLD develops. Furthermore, alternative fates of BCKA which are not oxidized in the liver of piglets fed MCFA should be examined. Finally, amino acid requirements (and nutrient requirements in general) of LBWT animals require more specific research based on superior protein utilization and fractional growth rates of LBWT neonatal pigs compared to NBWT pigs.

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