Examining the Influence of Muscle Fiber Type on Protein Turnover Signaling in Growing Pigs

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Keywords: pig, muscle fiber type, skeletal muscle, protein synthesis, protein degradation

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ABSTRACT

Postnatal skeletal muscle growth occurs through myonuclear accretion and high protein turnover rate. While fiber type composition of the muscle could affect protein turnover rate, less is known about how fiber type influences the regulation of protein synthesis and degradation signaling pathways. Thus, the hypothesis of this work was that variation in fiber type composition will differentially affect the regulation of signaling pathways related to protein turnover in skeletal muscle hypertrophy in growing pigs. Downregulated protein synthesis signaling and reduced expression of type II MyHC isoforms have been reported in skeletal muscles of low birth weight (LBWT) neonatal pigs. Therefore, we sought to determine whether these changes are sustained until weaning and would explain the reduction in LBWT pig growth compared to their normal birth weight (NBWT) sibling at weaning. Another objective was to determine whether the regulation of protein turnover signaling pathways are correlated to fiber type differences in skeletal muscles. Our data suggest that the longissimus dorsi (LD, glycolytic) muscle of LBWT pigs experienced compensatory growth while the soleus (oxidative) remained proportionally smaller. Growth of the LD was accompanied by upregulation of translation initiation. Additionally, there was no difference in expression of MyHC isoforms between NBWT and LBWT pigs. These data suggest the rapid growth of the LD of LBWT pigs may be attributed to an upregulation of protein synthesis signaling and occurred only in glycolytic muscles. A caveat in LBWT pig model is that the reduction in type II MyHC at birth is not the only factor that could influence muscle growth, and that other factors may have confounded our results. This is why we aimed to use β-adrenergic

agonist as a means to induce a shift fiber type in muscles to a more glycolytic phenotype. Our objective was to determine the influence of the β-adrenergic agonist Ractopamine (RAC) induced slow-to-fast fiber type transformation on the regulation of protein synthesis and degradation pathways. Although supplementation improved translational capacity, enhanced S6K1 phosphorylation, and reduced the abundance of calcium-dependent proteases, RAC feeding had no effect on body or muscle weights. These results suggest that a fiber type transformation without other physiological influences does not alter protein turnover signaling in favor of hypertrophy in growing pigs.

Keywords: pig, muscle fiber type, skeletal muscle, protein synthesis, protein degradation

GENERAL AUDIENCE ABSTRACT

Skeletal muscles grow by increasing the amount of protein contained within them. The amount of protein deposited is determined by the net balance between the rates at which proteins are synthesized and degraded. However, not all skeletal muscles grow at the same rate. One factor that is thought to influence protein synthesis and degradation rates is the types of muscle fibers that are present within a muscle. These fibers can display a range of contractile and metabolic characteristics, from slow-twitch oxidative fibers to fast-twitch glycolytic fibers. In the presented studies, we sought to determine whether changes in fiber type composition result in difference to the signaling pathways the regulate protein synthesis and degradation, ultimately leading to differences in the muscle growth of young pigs. We have previously shown reduced activation of the protein synthesis pathway in the skeletal muscle of low birth weight (LBWT) newborn pigs. These pigs also had lower expression of glycolytic fibers. In experiment 1, we aimed to compare the signaling pathways regulating protein synthesis and degradation in LBWT and normal birth weight (NBWT) pigs at weaning. We also sought to determine if the regulation of these signaling pathways changed between muscles with differing fiber type compositions. The glycolytic longissimus dorsi (LD) muscle of LBWT pigs grew rapidly between birth and weaning whereas the highly oxidative soleus did not. In addition, the LD of LBWT pigs had greater protein synthesis signaling and similar expression of muscle fibers compared with NBWT pigs, suggesting the improvement in protein synthesis signaling of LBWT pigs between birth and weaning may be related to a shift in fiber type. In experiment 2, we used a compound called ractopamine hydrochloride (RAC) to promote a slow-to-fast fiber type switch in the muscle of young pigs. With this study, we sought to determine the effect of this fiber type transformation, without the influence of birth weight, on the regulation of protein synthesis and degradation pathways.

Although RAC-fed pigs showed some minor changes that could improve protein synthesis and decrease protein degradation, RAC feeding had no observable effect on body weight or muscle growth. These results suggest that a fiber type transformation alone is not enough to promote muscle growth in growing pigs.

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Chapter 1. Introduction and Literature Review

INTRODUCTION

Protein synthesis and degradation, i.e. protein turnover, is a continuous and energy-demanding process that occurs in all tissues, including skeletal muscles (Welle and Nair, 1990). Protein turnover is controlled by various hormonal, nutritional and mechanical stimuli, protein turnover allows not only for regulating signaling pathways and remodeling of damaged tissues, but also for dictating the rate at which muscles grow or atrophy. For example, in growing animals elevated protein is needed for rapid skeletal muscle hypertrophy that is characteristic of early postnatal life (Davis and Fiorotto, 2009). However, even with the proper anabolic stimuli, not all muscles grow at the same rates. One factor that is thought to influence protein turnover rate in skeletal muscles is fiber type composition. Mammalian muscle fibers can be classified into four major fiber types (Type I, IIA, IIX, or IIB) by the myosin heavy chain (MyHC) isoform that is predominantly expressed by its myofibrils (Schiaffino and Reggiani, 2011). Skeletal muscles composed primarily of Type I, or slow-twitch oxidative fibers, have greater rates of protein turnover than those with primarily fast-twitch glycolytic, or Type IIX and IIB, fibers (Laurent et al., 1978; Bates and Millward, 1983; Kelly et al., 1984; Garlick et al., 1989). Although oxidative muscles can synthesize proteins at a much higher rate than glycolytic muscles, they are just as quickly degraded, thereby limiting muscles ability to accrete protein.

While there is an abundance of studies that demonstrate a difference in protein turnover rates across muscles of varying fiber type compositions, less is known about the fiber type-related differences in regulation of the catabolic and anabolic signaling pathways contributing to protein metabolism in growing animals. Thus, the overall hypothesis of this project was that variations in fiber type composition will differentially influence the regulation of signaling pathways related to

protein synthesis and degradation. Such changes will ultimately lead to differences in skeletal muscle hypertrophy in growing pigs. The objectives of this research project were to: 1) compare protein turnover signaling in LBWT and NBWT pig skeletal muscle at weaning, 2) investigate whether protein turnover signaling differs in skeletal muscles with different fiber type compositions, 3) determine whether β-adrenergic agonist supplementation would enhance MyHC-IIB expression in nursery-age pigs, and 4) evaluate the influence of a slow-to-fast fiber type transformation on the regulation of protein synthesis and degradation pathways.

LITERATURE REVIEW

Myogenesis

Skeletal muscles, which allow for voluntary movement and maintenance of posture, are composed of multinucleated cells called myocytes. The formation of myocytes, commonly known as muscle fibers or myofibers, proceeds in a biphasic fashion. The first phase of myogenesis, known as primary myogenesis, occurs between days 35 and 55 of gestation in pigs (Wigmore and Stickland, 1983). This stage begins with the proliferation and differentiation of multipotent myogenic progenitor cells into embryonic myoblasts (Yan et al., 2013). Following the appropriate environmental signals, embryonic myoblasts migrate and fuse to form elongated structures with multiple centrally-located nuclei, called primary myotubes (Du et al., 2010). By day 55 post-conception, a second population of myoblasts begins to proliferate among the primary myotubes (Wigmore and Stickland, 1983), signaling the transition to secondary myogenesis. During this phase, fetal myoblasts use the nascent primary myotubes as scaffolding to progressively align and fuse to form a secondary generation of myotubes (Wigmore and Stickland, 1983). Following innervation of the primary myotube, secondary myofibers separate from the primary myotube and form their own basal lamina (Kelly and Zacks, 1969; Harris and Harris, 1981). The number of secondary myocytes generated from each primary myotube is typically around 20:1 in pigs (Stickland and Handel, 1986; Božičković et al., 2017), but varies based on factors such as whole muscle size, breed, and maternal nutrition (Stickland and Handel, 1986; Dwyer et al., 1994). This process is complete between days 90 to 95 of gestation (Lefaucheur et al., 1995a), thus the total fiber number is fixed prior to birth.

An important step in myofiber maturation is the assembly of myofibrils which are the rod-like structures that contain the contractile machinery of skeletal muscle. Under a microscope,

myofibrils have a striated appearance due to segments of light and dark repeating units known as sarcomeres (Gautel and Djinovic-Carugo, 2016). Each sarcomere consists of structural proteins and interdigitating myofilament chains that allow for contraction and force generation within a myocyte (Gerrard and Grant, 2003). The myofilaments, myosin (dark) and actin (light), are held in an overlapping fashion by the structural proteins, termed the sarcomeric cytoskeleton (Gautel and Djinovic-Carugo, 2016). In the presence of calcium and ATP, the globular heads of myosin bind to the actin filament and undergo conformational change that drags the Z-lines, or lateral demarcations of the sarcomere, toward the M-line, or center of the sarcomere (Kraft et al., 2005). This process results in the shortening of sarcomeric length (crossbridge cycling), contraction of the muscle fiber, and ultimately contraction of the whole muscle (Fitts, 2008). While this mechanism is well conserved across species and across different skeletal muscles, the speed and force of contraction can vary in response to a multitude of factors. One significant aspect influencing this variability is the isoform of myosin present within the myofibril (Reiser et al., 1985; Geiger et al., 2000). This allows for the classification of different muscle based on their "fiber types".

Muscle Fiber Types

The myosin filament (filamentous myosin) consists of 300-400 molecules of myosin proteins, which self-associate to form a chain (Lodish et al., 1995). Myosin protein, an asymmetrical heterohexamer, consists of four myosin light chains (MLCs) and two myosin heavy chains (MyHCs) (Shrager et al., 2000). These light and heavy chains interact to form two major structures: a rod-like tail domain and a globular head (Lodish et al., 1995). While the tail domain is crucial in the formation and stability of filamentous myosin, the globular head is the site of actinomyosin interaction (Shrager et al., 2000; Schiaffino et al., 2015). More specifically, it is the

MyHC monomers which make up the bulk of the globular head and that possess the enzymatic active sites necessary for ATP hydrolysis and bind with actin. MyHCs belong to a family of actin-based motor proteins with the ability to convert chemical energy into mechanical force (Weiss et al., 1999). There are currently seven distinct classes of MyHC, categorized by the properties of their head domain (Weiss and Leinwand, 1996). In striated muscle, the various isoforms of Class II MyHC, also known as sarcomeric MyHC, determine the speed, strength, and bioenergetic cost of contraction (Weiss et al., 1999). It is important to note that a myofiber may express many isotypes of sarcomeric MyHC throughout its lifetime and, in some cases, simultaneously (Staron and Pette, 1993; Kim et al., 2014). Furthermore, expression may vary in response to a multitude of factors, such as organism life stage**,** genetic propensity for muscling, athletic training, and disease (Short et al., 2005; Strbenc et al., 2006; Wimmers et al., 2008; Ciciliot et al., 2013).

Porcine skeletal muscles exhibit a dynamic combination of six MyHC isoforms: MyHC-embryonic, MyHC-neonatal, MyHC-slow, MyHC-IIA, MyHC-IIX, and MyHC-IIB (Picard et al., 2002). The two developmental isoforms, MyHC-embryonic and MyHC-neonatal, are transiently expressed in nascent myotubes and only resurface during times of muscle regeneration (Narusawa et al., 1987; Schiaffino et al., 2015). The "adult" isoforms, MyHC-slow, MyHC-IIA, MyHC-IIX, and MyHC-IIB, ultimately replace the developmental isoforms at various stages of fetal and postnatal development in response to neural and hormonal stimuli (Gambke et al., 1983). Depending on the composition of isoforms expressed, as well as the presence or absence of specific metabolic enzymes, each fiber will fall into one of four muscle "fiber types". These types differ in categories such as color, speed of contraction, mature size, fatigue resistance, and metabolism preference. Primary muscle fibers, which begin to synthesize MyHC-slow soon after formation, typically mature into type I muscle fibers (Wank et al., 2006). Type I fibers, also known as "slow-

twitch" fibers, are characterized by slow contraction velocities, high fatigue resistance, exclusively oxidative metabolism, and deep red color due to high myoglobin content and abundant vascularization (Ordway and Garry, 2004). This fiber type is commonly seen in postural or masticatory muscles and allows for prolonged stints of aerobic activity (Toniolo et al., 2004). Secondary myofibers, on the other hand, have greater variability in the fiber types they may become. During late fetal development, a small population of secondary fibers in close proximity to a primary fiber may begin to express MyHC-slow, later maturing into additional type I fibers (Gerrard and Grant, 2003). Another subpopulation may prenatally express MyHC-IIA and eventually become type IIA fibers. Type II fibers, also called "fast-twitch" fibers, are characterized by rapid contraction velocities. The average diameter of type II myofibers is also considerably larger than type I fibers, likely due to a lesser dependence on oxygen and exogenous metabolic substrates (Ashmore et al., 1972). Type IIA fibers, compared to the other fast fiber types, are the most similar to type I fibers. This group has the slowest contraction velocity of the fast fiber type, are relatively fatigue resistant, and possess a high oxidative capacity (Toniolo et al., 2004). On the periphery of a muscle bundle, any secondary myofibers not expressing MyHC-slow or MyHC-IIA after birth will synthesize one of the remaining fast isotypes, MyHC-IIX or MyHC-IIB (Picard et al., 2002). Type IIB muscle fibers have the fastest contractions speeds of all fast fibers and derive their energy supply from the glycolytic pathway (Zierath and Hawley, 2004). These fibers may also be identified by having the largest mature size, as well as by their pale pink-white color. Type IIX myofibers are intermediate in most categories compared with type IIA and type IIB fibers. This type is classified by intermediate size, color, and contraction speed, as well as the capacity for both oxidative and glycolytic metabolisms (Lefaucheur et al., 1998; Gerrard

and Grant, 2003). This formation of slow fibers surrounded by a ring of fast fibers results in a "rosette" pattern, an appearance that is unique to porcine skeletal muscle (Lefaucheur et al., 1998).

Although the composition of fiber types are relatively homogenous across most neonatal skeletal muscles, this does not imply that muscle fiber compositions will not change (Wank et al., 2006). Myofibers must retain some plasticity in order to accommodate the functional demands of the muscle, as well as adapt to environmental insults (Gunawan et al., 2007). This occasionally leads to the presence of "hybrid" fibers, or fibers which express more than one MyHC protein. It is worth mentioning that co-expression of MyHC isoforms is less prevalent at the mRNA level than at the protein level (Lefaucheur et al., 2002), suggesting that myofibrils typically express the gene for one isoform at a time and are in the process of transitioning to the isotype of the gene being expressed. As fiber type transformations shift along an obligatory path (I ↔ IIA ↔ IIX ↔ IIB), the hybrid fibers, type I-IIA, IIA-IIX, and IIX-IIB fibers, are thought to be transitional isoforms which bridge the gaps between "pure" fiber types (Pette and Staron, 1997; Pette, 2002).

Postnatal Growth

The early postnatal period is a critical time where neonates must adapt from the intrauterine to extrauterine environment. Part of their transition includes a dramatic increase in growth rate, where healthy piglets may triple or quadruple their body weight within the first three weeks of life (Wood and Groves, 1965; Milligan et al., 2001). This weight gain is largely due to the rapid growth of skeletal muscle, which accounts for a majority of the carcass weight in finished pigs (Carr et al., 1978). Mature skeletal muscle size is determined not only by the total number of muscle fibers present, but also by their variable cross-sectional areas (Dwyer et al., 1993). Considering that total fiber number is fixed at birth for most mammalian species, postnatal muscle growth occurs almost exclusively through the hypertrophy of existing fibers. This phenomenon is

mediated by the action of a third and final population of multipotent myogenic cells (Charge and Rudnicki, 2004; Bruusgaard et al., 2010). These mononucleated muscle stem cells, known as "satellite cells" for their peripheral location between a myofiber and its associated basal lamina (Mauro, 1961), are crucial for postnatal muscle growth, as well as regeneration in response to injury (Yin et al., 2013). Typically found in greater abundance in oxidative myofibers (Gibson and Schultz, 1982), satellite cells remain mitotically quiescent until stimulated by one of many activators, including mechanical stretch, myofiber cytokines, and various growth factors and hormones (Pallafacchina et al., 2013; Dayanidhi and Lieber, 2014). Post-activation, they undergo asymmetric divisions which produce differentiated myoblasts, as well as new satellite cells that return to quiescence (Pallafacchina et al., 2013). The nascent myoblasts then fuse with existing muscle fibers or, in cases of severe tissue damage, generate new myotubes (Fu et al., 2015). Through this fusion, satellite cells provide additional myonuclei to their associated fiber, thereby increasing its transcriptional capacity (Bruusgaard et al., 2010). Furthermore, satellite cells also contribute other organelles, including abundant free and membrane-bound ribosomes (Schultz, 1976). Satellite cell fusion allows for a surge in the protein synthetic rate (Fiorotto et al., 2014) by increasing the DNA transcriptional capacity, as well as providing additional ribosomes to serve as the protein translational machinery.

Any change in muscle mass is indicative of a shifted balance between two carefully regulated processes: protein synthesis and protein degradation. These opposing processes, which jointly control the continuous renewal of proteins, determine net protein accretion or loss (Toyama and Hetzer, 2013). For example, when the rate of protein synthesis outpaces the rate of degradation, as is the case in neonatal skeletal muscles (Davis and Fiorotto, 2009; Suryawan and Davis, 2014), the balance is tipped in favor of protein deposition and growth of the muscle fibers.

However, this temporary elevation in the synthetic rate progressively subsides with age and will eventually reach an equilibrium with the rate of protein degradation at maturity (Davis and Fiorotto, 2009). After reaching maturity, the rate of protein synthesis continues to slow to a point where the rate of degradation surpasses that of protein synthesis (Gonskikh and Polacek, 2017). When the amount of proteins synthesized becomes less than the amount being lost through degradation, the consequence is an age-related atrophy of muscle fibers. Interestingly, the overall rate of protein turnover, which varies by tissue, also varies between the glycolytic and oxidative muscle phenotypes. While oxidative muscles are known to exhibit greater rates of protein synthesis than glycolytic muscles (Laurent et al., 1978; Bates and Millward, 1983; Kelly et al., 1984), this is thought to be balanced with a higher rate of degradation (van Wessel et al., 2010).

Protein Synthesis

Protein synthesis, through messenger RNA (mRNA) translation, is the pathway in which cells assemble amino acids necessary to generate specific proteins. This fundamental process requires a strand of mRNA to be translated, a translationally-competent ribosome, amino acid specific transfer RNAs (tRNAs), and a sufficient supply of amino acids (Cooper and Hausman, 2013). Translation of mRNA to proteins utilizes approximately 20% of available cellular ATP (Buttgereit and Brand, 1995), and occurs in three highly regulated steps: initiation, elongation, and termination (Roux and Topisirovic, 2012).

Translation Initiation. In eukaryotes, translation initiation is the first and most extensively regulated step of mRNA translation and ends with the formation of a translationally-competent ribosome. This process can be broken down into 3 phases. First, the 43S pre-initiation complex is formed by the association of the small ribosomal component (40S) with a number of accessory proteins called eukaryotic initiation factors (eIFs) (Bhat et al., 2015). In this phase, eIF2 binds

with GTP and delivers the initiator methionyl tRNA (Met-$tRNA_i$) to the complex composed of 40S subunit, eIF1, eIF3, and eIF5 (Farruggio et al., 1996), forming the 43S pre-initiation complex. Next, eIF4F guides a strand of mRNA to the 43S pre-initiation complex, where the pre-initiation complex can then bind to the cap structure at the 5' end of mRNA (Sonenberg and Hinnebusch, 2009). Finally, the pre-initiation complex scans along the mRNA strand until the AUG start codon is located (Sonenberg and Hinnebusch, 2009). AUG recognition triggers dissociation of all initiation factors (Kolupaeva et al., 2005) and allows 60S to join the 40S subunit on the mRNA strand, thus completing the assembly of the translational machinery.

Regulation of Translation Initiation. There are many pathways which, when stimulated, contribute to translation initiation. One highly conserved pathway is the PI3K-AKT-mTOR signaling cascade. This mechanism regulates translation following the binding of extracellular ligands, specifically insulin and various growth factors, to their surface receptor (Roux and Topisirovic, 2012). This stimulates the activation of phosphoinositide 3-kinase (PI3K), which is responsible for the conversion of phosphatidylinositol 4,5-bisphosphate (PIP_2) into phosphatidylinositol 3,4,5-triphosphate (PIP_3) (Engelman et al., 2006). PIP_3 binds both phosphoinositide-dependent kinase 1 (PDK1) and AKT, where PDK1 will phosphorylate and activate AKT (Engelman et al., 2006). Once phosphorylated, AKT inhibits the tumor suppressor complex 2 (TSC2) that functions as a mammalian target of rapamycin complex (mTORC1) inhibitor (Ma and Blenis, 2009). mTORC1 has two major downstream targets that regulate protein synthesis: eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and ribosomal protein S6 kinase (S6K1) (Bodine, 2006). Activated mTORC1 phosphorylates multiple residues on 4E-BP1, causing its dissociation from eukaryotic initiation factor 4E (eIF4E), thus allowing eIF4E to then complex with eukaryotic initiation factor 4G (eIF4G) (Richter and Sonenberg, 2005). The newly

formed eIF4E:eIF4G complex recruits the RNA helicase eukaryotic initiation factor 4A (eIF4A) to form eIF4F, the heterotrimeric complex necessary for the binding of mRNA to the pre-initiation complex (Roux and Topisirovic, 2012). The other downstream effector, S6K1, also contributes to the initiation of translation once activated by mTORC1 (Roux and Topisirovic, 2012). Major downstream targets of S6K1 include programmed cell death protein 4 (PDCD4) and eukaryotic initiation factor 4B (eIF4B) (Raught et al., 2004; Dorrello et al., 2006). PDCD4 interferes with the formation of eIF4F through binding with eIF4A (Raught et al., 2004; Dorrello et al., 2006). Activation of S6K1 not only stimulates the activity of eIF4B, which promotes the helicase activity of eIF4A, but also results in the degradation of PDCD4, freeing eIF4A to participate in formation of eIF4F (Raught et al., 2004; Dorrello et al., 2006).

Peptide Elongation. The next phase in protein synthesis is peptide elongation. Ribosomes possess three adjacent t-RNA binding sites, known as the acceptor (A) site, a peptidyl (P) site, and an exit (E) site (Uemura et al., 2010), each aligning with a mRNA codon. Following successful AUG recognition during the initiation phase, Met-$tRNA_i$ is shifted from its original position in the ribosomal A site to the adjacent P site, thus sliding the next codon to be decoded into alignment with the A site (Dever and Green, 2012). This now vacant A site is ready to receive another aminoacyl-tRNA. Eukaryotic elongation factor 1A (eEF1A) binds to aminoacyl-tRNAs and guides them to the A site, at which point eEF1A is released by GTP hydrolysis following codon recognition (Dever and Green, 2012). Once situated, the P site aminoacyl-tRNA transfers its amino acid to the A site tRNA through the formation of peptide bonds, forming a polypeptide chain (Dever and Green, 2012). The ribosome again advances to the next codon, shifting the deacylated tRNA to the E site, while the adjacent peptidyl-tRNA moves into the P site (Uemura et al., 2010). Finally, the deacylated E site $tRNA_i$ is quickly release while another aminoacyl-tRNA

can enter the A site (Uemura et al., 2010). This process is repeated, adding additional amino acids with each codon translated, until a stop codon is encountered.

Translation Termination. The short termination phase of protein translation is triggered when a stop codon, identified by codons containing UAA, UGA, or UAG, aligns with the ribosomal A site. Due to the fact that cells do not produce tRNAs with anticodons that complement these stop codons, the elongation of the polypeptide chain is brought to a halt (Cooper and Hausman, 2013). However, cells do produce other proteins, called release factors (RFs), that do recognize these signals for translation termination. In both eukaryotes and prokaryotes, there are two classes of RFs whose combined actions ultimately lead to the dissociation of the polypeptide chain from the ribosome (Zhouravleva et al., 1995). In eukaryotes, these release factors are eukaryotic release factor I (eRF1, class I) and eukaryotic release factor 3 (eRF3, class II), which bind to form a ternary termination complex with GTP (Frolova et al., 1996; Frolova et al., 2000). When a stop codon is encountered, eRF3 guides the complex to the ribosome while eRF1 recognizes and binds the codon (Dever and Green, 2012). Once activated through its complexing with eRF1 and the ribosome, the GTPase eRF3 cleaves the bond between the completed polypeptide chain and its tRNA (Frolova et al., 1996). Finally, the translational machinery, including ribosomal subunits, release factors, and tRNAs, dissociate from the mRNA (Cooper and Hausman, 2013).

Protein Degradation

The other process that the rate of protein turnover is the rate of degradation. The degradation of proteins is a continuous process allowing for recycling of substrates, quality control (eliminates faulty or damaged proteins), and quick adaptation to changing physiological conditions. Failure to maintain appropriate degradation has been implicated in many diseases, as

well as some aspects of aging (Toyama and Hetzer, 2013; Hanna et al., 2019). In eukaryotes, this important regulatory function is carried out in large part by selective degradation through the Ubiquitin-proteasome pathway (UPP) (Lecker et al., 2006). The UPP, by which most intracellular proteins are degraded (Rock et al., 1994), uses a 8.6 kDa co-factor called ubiquitin to flag specific proteins for degradation (Cooper and Hausman, 2013). It is critical for cells to maintain an adequate supply of free ubiquitin considering the heavy dependence eukaryotes place on the UPP for protein degradation. This is accomplish through *de novo* synthesis of ubiquitin precursors, as well as recycling of ubiquitin following degradation of the target protein (Grou et al., 2015). In the case of *de novo* synthesis, ubiquitin can initially be found attached to a ribosomal protein or as a polymer of ubiquitins linked in a head-to-tail fashion (Cooper and Hausman, 2013; Grou et al., 2015). A particular class of hydrolases, known as deubiquitinases (DUBs), are required to release monomeric ubiquitin from these precursory forms, in addition to disassembling ubiquitin chains for recycling (Grou et al., 2015). Once released, monomeric ubiquitin can then be utilized in the Ubiquitin-proteasome pathway.

Protein Ubiquitination. The first step in ubiquitin-dependent proteasomal degradation is ubiquitination, or the process by which ubiquitin is attached to a target protein through a series of enzymatic reactions (Cooper and Hausman, 2013). This process begins with the activation of ubiquitin by the aptly-named ubiquitin-activating enzyme (E1). In the presence of free ubiquitin, E1 binds ubiquitin through the formation of a reactive thiolester bond (Lee and Schindelin, 2008). This activated ubiquitin-thiolester can then be transferred from E1 to a second class of enzyme, the ubiquitin-conjugating enzymes (E2) (Stewart et al., 2016). The ubiquitin-burdened E2 enzyme is subsequently recruited by a ubiquitin ligase (E3), bringing E2 in close proximity to the E3 target protein. E3 ligases facilitate the transfer of ubiquitin from E2s to the target protein (Clague and

Urbe, 2010), although there is some evidence E2 enzymes also have the ability to ubiquitinate proteins (Stewart et al., 2016). This process is repeated and forms a polyubiquitin chain, the degradative signal recognized by the proteasome. An interesting feature of this mechanism is the inverse relationship between enzyme abundance and selectivity of targets. For example, the least abundant E1 enzyme can interact with any E2 enzyme isoform. In contrast, each of the 100 or so isoforms of E2 enzymes, being semi-selective, may only interact with a small selection of E3 ligases. The most selective, however, are the E3 ligases, which number in the thousands but are only compatible with a small number of E2 isoforms and even fewer target proteins. This highly specific nature of E3 ligases allows for extreme control over protein abundance through the regulation of ligase translation by the cell.

Proteasomal Degradation. Once recognized, the degradation of polyubiquitinated proteins is carried out by the 26S proteasome complex. This large complex is at least 50 times larger than most other proteases and is composed of a barrel-shaped 20S protease with a 19S regulatory cap on either end (Kim et al., 2011). Located on the 19S caps are six ATPases that function to bind and unfold globular proteins, as well as assist in directing the linearized protein into the proteasomal lumen for digestion (Clague and Urbe, 2010; Kim et al., 2011). There, the protein is digested into small peptides while the polyubiquitin chain, which is cleaved by DUBs upon binding of the protein to the proteasome, is disassembled for reuse.

Regulation of the Ubiquitin-Proteasome Pathway. As previously mentioned, protein degradation can be stimulated for a number of conditions, including to alter gene transcription through degradation of transcription factors, to eliminate damaged or misfolded proteins, to provide amino acids for gluconeogenesis during fasting, and to allow for tissue remodeling (Kaloyianni and Freedland, 1990; Dhananjayan et al., 2005; Hesselink et al., 2006; Marques et al.,

2006; El-Kadi et al., 2018a). Thus, the major mechanisms for degradation need to be amenable to regulation by intra- and extra-cellular signals. For example, the UPP can be regulated through the same signaling cascade that regulates protein synthesis. While the activation of AKT stimulates protein synthesis through its subsequent activation of mTOR, activated AKT simultaneously downregulates protein degradation through phosphorylation, and thus inactivation, of the forkhead box O (FoxO) transcription factors (Stitt et al., 2004). However, when unphosphorylated as a result of decreased AKT activity, activated FoxO transcription factors shed their inhibitory chaperone proteins and translocate to the nucleus, where they promote the transcription of atrophy-related genes, commonly referred to as atrogenes (Sandri et al., 2004; Huang and Tindall, 2007). These atrogenes include the skeletal muscle-specific E3 ligases, muscle atrophy F-Box protein (Atrogin-1) and muscle RING finger protein 1 (MuRF1) (Kandarian and Jackman, 2006; Toyama and Hetzer, 2013).

Calpain system. Another system contributing to the regulation of skeletal muscle protein degradation is the calpain system. This system is comprised of multiple calcium-dependent cysteine proteases, called calpains, as well as their regulatory components. (Sorimachi and Ono, 2012). The members of this protease family are typically classified as "conventional" or "unconventional" calpains based on their expression profile (Ono and Sorimachi, 2012). The conventional calpains, calpain-1 and calpain-2, are ubiquitously expressed across mammalian tissues while the unconventional calpains are tissue-specific (Ono and Sorimachi, 2012). One way the calpain system differs from most other proteases is that they serve in a processing capacity rather than a truly degradative one (Sorimachi and Ono, 2012). For instance, the ubiquitin-dependent degradation of myofibrillar proteins is very slow when proteins are associated with the actomyosin complex, likely due to inter-protein interactions that stabilize the complex (Solomon

and Goldberg, 1996). The calpain family facilitates this degradation process through fragmentation of myofibrillar structural proteins (Goll et al., 2003). The resulting dismantled sarcomeric scaffold releases protein fragments that can then be digested by the proteasome (Sorimachi and Ono, 2012). The calpain system has two regulatory components that influence calpain activity: calpain small subunit 1 (CAPNS1) and calpastatin. The first component, CAPNS1, is a regulatory subunit that forms a dimer with the larger catalytic subunits, calpain-1 (CAPN1) and calpain-2 (CAPN2) (Yoshizawa et al., 1995; Ono and Sorimachi, 2012). While CAPNS1 does not directly contribute to the proteolytic activity of the calpain dimers, its function as a chaperone protein is vital to the stability of these conventional calpains (Yoshizawa et al., 1995).

The other regulatory component, calpastatin, is the sole endogenous inhibitor of the calpain system (Kiss et al., 2008). Calpastatin has four inhibitory sites that can simultaneously bind calpain proteins (Hanna et al., 2007). Inhibition of calpain activity prevents the fragmentation of myofibrillar proteins, thus reducing the total protein degradation in skeletal muscle. Interestingly, calpastatin inhibits both conventional and unconventional calpains, with the exception of skeletal muscle-specific CAPN3, or calpain-3 (Ono et al., 2004). Calpain-3, acting as the gate-keeper of sarcomeric protein turnover, can also degrade calpastatin in order to preserve the fragmentation activity of the conventional calpains (Kramerova et al., 2005; Beckmann and Spencer, 2008). This function, which promotes the removal of damaged or atrophy-induced proteins, is vital to the maintenance and growth of healthy muscle (Kramerova et al., 2005).

Intrauterine Growth Restriction

It is well established that the genome has powerful influence over many valuable traits, however, genotype alone is not enough to guarantee that an individual will attain their maximum

growth or performance potential. This is because nutrition, hormonal influences, and various other environmental factors interact with the genetic potential to determine the resulting phenotype. This concept of phenotypic plasticity, or "the change in the expressed phenotype of a genotype as a function of the environment" (Scheiner, 1993), is the basic principle behind the study of developmental programming, where intrauterine and neonatal environments influence physiological function and wellbeing in adulthood (Hales and Barker, 2001; Langley-Evans, 2006). One such example of developmental programming occurs in response to intrauterine growth restriction (IUGR). By definition, IUGR is the term used to describe a negative deviation from expected fetal growth *in utero,* and is a leading cause of perinatal morbidity and mortality in humans and livestock species (Chapple and Mann, 2018). This condition can arise from a variety of prenatal complications such as placental insufficiency, maternal undernutrition, intrauterine crowding, fetal endocrine imbalances, and disease (Pardo et al., 2013; Salam et al., 2013; Gaccioli and Lager, 2016; Sharma et al., 2016).

In today's livestock production, the prenatal environment for pregnant dams and their offspring regularly competes with financially-driven management decisions. These include breeding younger, often immature females, as well as the persistent genetic selection for larger litter sizes in polytocous species like the pig. While maternal undernutrition in livestock is typically not an issue with good herd management, breeding younger females or selecting for larger litters inadvertently escalates the competition for other limited maternal resources, such as uterine space or blood supply (Reynolds and Caton, 2012). For example, there is a particularly antagonistic relationship between a sow's litter size and the probability of producing IUGR-affected piglets (Rutherford et al., 2013). As the number of piglets in a litter increases, fetuses may become confined between littermates and, as a result, compete for a smaller portion of the

uterine horn in which to occupy. This limits the surface area for placental contact, therefore restricting the nutrients the fetus receives and can ultimately manifest in IUGR (Argente et al., 2008).

After birth, IUGR neonates are primarily recognized by low birth weights (LBWT). IUGR-afflicted individuals are classically defined as being below the 10^{th} percentile for gestational age in humans or, in litter bearing species, by weighing less than 1.5 standard deviations below the mean litter birth weight (Cohen et al., 2015; Matheson et al., 2018). In infants and piglets, LBWT is associated with greater postnatal morbidities and mortality (Matheson et al., 2018), as well as a greater risk for metabolic issues arising later in life (Hales and Barker, 2001; Shen et al., 2018). Additionally, IUGR in infants and piglets causes asymmetrical growth and development of tissues also known as the "brain sparing effect" (Bauer et al., 1998). During prenatal oxygen or nutrient deprivation, fetal hemodynamics are altered through the strategic restriction or dilation of blood vessels, in order to increase the likelihood of the fetus surviving until birth (Tchirikov et al., 1998; Hernandez-Andrade et al., 2008). This preferential shunting follows a hierarchical order of nutritional priority that determines the volume of blood, and therefore nutrients, that organ will receive. The brain, with the greatest impact on survival, is the main target for nutrient delivery and therefore experiences the least effects on perfusion. This is followed by viscera, such as the liver and gastrointestinal tract, which experience a mild deprivation of nutrients (Turan et al., 2008). Finally, tissues such as adipose and skeletal muscle experience the greatest reduction in perfusion, likely owing to their relative disuse before birth. (Lefaucheur et al., 2003). By directing vital nutrients away from skeletal muscles, even temporarily, the brain-sparing effect not only contributes to the characteristic small body size associated with IUGR at birth, but also initiates a

cascade of physiological changes in skeletal muscle, which have both immediate and long-term implications on the growth and wellbeing of the individual.

IUGR and Myogenesis. The influence of fetal nutrient deprivation on myogenesis greatly depends on when the insult occurs during gestation. For instance, nutrient deprivation during late gestation may reduce muscle fiber cross sectional area but leaves total fiber number generally unaffected (Greenwood et al., 1999). However, if the insult occurs earlier in gestation, as is the case for many IUGR individuals, fetal undernutrition instead hinders the initial formation of muscle fibers. This can lead to reduced total fiber number, altered fiber type composition, and ultimately reduced whole muscle weights (Ward and Stickland, 1991; Yates et al., 2012). During myogenesis, primary myotubes from growth-restricted porcine fetuses exhibit smaller myofibril-free regions, which reduces their overall diameter (Wigmore and Stickland, 1983; Almeida et al., 2019). As the diameter of primary myotubes is reduced, there is a corresponding reduction in the available surface area to serve as scaffolding, thus limiting the number of secondary myofibers that can be formed during secondary myogenesis (Wigmore and Stickland, 1983). Not only does this reduction in fiber density limit the muscle's potential growth, but it also hinders secondary fiber formation, which reduces the population of fast-twitch myofibers that could adopt a glycolytic phenotype at maturity (Lefaucheur et al., 1995b) and results in a shift towards a more oxidative phenotype at the whole muscle level in LBWT neonates (Wank et al., 2000; Lefaucheur et al., 2003). Additionally, skeletal muscles in LBWT piglets express emb-MyHC much longer than their normal birth weight (NBWT) siblings, suggesting that maturation of muscle fibers is delayed in LBWT piglets (Almeida et al., 2019). This theme of delayed maturity is also reported for the changes to the insulin signaling pathway in LBWT skeletal muscles.

Insulin-like growth factors (IGFs) have a unique role in skeletal muscle growth in that they stimulate both myoblast proliferation and differentiation through the same receptor (Coolican et al., 1997). While these processes are thought to be antagonistic, the promotion of one or the other depends on the availability of oxygen (Brown and Hay, 2016). In hypoxic conditions, the anabolic hormone insulin-like growth factor-1 (IGF-I) promotes the proliferation of myoblasts, even when they would typically be undergoing differentiation under normoxic conditions (Ren et al., 2010). Given that both oxygen deprivation (Brown and Hay, 2016) and a reduction in circulating IGF-I concentrations (Soto et al., 2017) are common in IUGR, it is possible that myoblasts of growth-restricted fetuses are not only prevented from differentiating but and proliferating. In fact, primary myoblasts of IUGR fetal lambs have slower proliferation rates compared with those from control lambs, resulting in fewer differentiated myoblasts (Yates et al., 2014). This suggests that skeletal muscles of growth-restricted fetuses appear smaller and less mature because they fall behind the developmental trajectory seen in unaffected fetuses. Through the restriction of secondary myofiber formation and hindrance of normal myocyte development, IUGR not only promotes the characteristically smaller muscle weights, but also results in skeletal muscle with a more fetal-like appearance.

IUGR and Postnatal Growth. Compromised skeletal muscle growth during gestation is a major contributor to the characteristic appearance of LBWT offspring. However, this is only one of the physiological differences that exist between offspring receiving proper nutrition *in utero* and those who do not. For example, LBWT piglets are less efficient at converting feed into lean tissue, are older when they reach an acceptable market weight, and possess less desirable carcass qualities than their NBWT counterparts (Gondret et al., 2006; Zhang et al., 2018). These characteristics often pose a problem in commercial meat production, where LBWT pigs not only

require more resources to reach market weight, but also return less profit for their finished products. A similar trend of impaired growth and altered body composition is reported in LBWT infants, in addition to having an increased risk of developing metabolic disorders in adulthood, such as hypertension and insulin resistance (Chakraborty et al., 2007; Jornayvaz et al., 2016). Although it is not uncommon for LBWT neonates to remain smaller throughout their lifetime (Crume et al., 2014), most undergo compensatory growth, where LBWT individuals exhibit a period of accelerated postnatal growth (Chakraborty et al., 2007). However, it is important to note that this "catch-up growth" is largely due to increased fat deposition (Ibanez et al., 2011; Cho and Suh, 2016) rather than the stimulation of muscle hypertrophy, thereby increasing the likelihood of metabolic disorders in later in life (Loos et al., 2001, 2002; Ezzahir et al., 2005).

IUGR and Protein Turnover. The diversion of nutrients away from skeletal muscle during IUGR causes not only immediate nutrient deprivation but also initiates physiological changes. While these physiological changes reduce fetal nutrient demands in the short term, they often result in long-term impairments to protein accretion. For instance, myofibers from LBWT lambs contain fewer myonuclei compared with those born NBWT (Greenwood et al., 1999). While it may require less energy to maintain a smaller population of satellite cells, this permanent modification limits the transcriptional ability of the tissue, thereby reducing its lifetime translational capacity. In addition, LBWT can hinder protein synthesis is through changes to the insulin signaling pathway. In mammals, insulin and insulin-like growth factors play an important role in communicating nutrient availability and directing enzymatic activities across multiple tissues (White and Kahn, 1994). IGF-I, which was previously mentioned for its involvement in delaying myoblast differentiation, is critical to the activation of protein synthesis and can be used as an indicator of nutritional status (Ketelslegers et al., 1995; Lang and Frost, 2004). IGF-I acts as a ligand for

insulin receptor (IR) and insulin-like growth factor-1 receptor (IGF-IR) (Nakae et al., 2001). Once activated IR and IGF-IR phosphorylate and activate insulin receptor substrate-1 (IRS-1). IRS-1 then activates PI3K, thus providing a direct link between nutrient availability and the PI3K-AKT signaling pathway (Backer et al., 1992). In LBWT neonatal pigs, circulating concentrations and skeletal muscle expression of IGF-I are reduced compared with those of NBWT siblings (Chen et al., 2017). It then follows that lower IGF-I availability in LBWT neonate contributes to the reduction in insulin-dependent activation of the PI3K/AKT pathway and initiation of protein synthesis (Jensen et al., 2008). Interestingly, both mRNA expression and protein abundance of IGF-IR are typically elevated in LBWT animals (Muhlhausler et al., 2009; Iñiguez et al., 2014; Chen et al., 2017), although this is likely to be a compensatory response for low IGF-I concentrations.

Since the downregulation of the PI3K-AKT pathway is known to lessen the rate of protein synthesis, it stands to reason that this would also hold implications for the protein degradation pathways. Due to the role of AKT as a central signaling node for both protein synthesis and degradation, a downregulation in the anabolic signal would diminish AKT activity, allowing for greater FOXO transcription factor activation and upregulated transcription of atrogenes. In fact, chemically-induced atrophy in cultured myotubes and mouse models results in reduction in AKT and S6K phosphorylation, as well as greater expression of one or both ubiquitin ligases (Sandri et al., 2004; Stitt et al., 2004). While it seems logical that a similar process may cause the impaired growth of LBWT skeletal muscles, it has yet to be fully ascertained what influence LBWT possesses over the rate of protein degradation. For the moment, recent work in LBWT piglets suggests the major protein degradation pathways remain relatively unchanged (El-Kadi et al., 2018b), however further investigation is needed.

β-adrenergic Agonists

β-adrenergic agonists have long been used to treat airway disorders, such as chronic obstructive pulmonary disorder (COPD) or asthma, by promoting relaxation in the smooth muscle of respiratory airways (Cazzola and Matera, 2009; Barisione et al., 2010). These sympathomimetic agents share a similar structure to endogenous catecholamines and exert their actions through the adrenergic signaling pathway (Billington et al., 2017). Upon binding of the β-agonist with its receptor, the activated adrenoceptor interacts with an associated G-protein complex, consisting of a stimulatory α-subunit and two smaller β- and γ-subunits. This results in the activation of the α-subunit, which triggers its dissociation from the βγ-subunits. The free α-subunit can then migrate to and phosphorylate adenylate cyclase, an enzyme responsible for converting ATP to cAMP. This subsequent accumulation of cAMP activates protein kinase A (PKA), which interacts with a variety of downstream targets (Billington et al., 2017).

Aside from its success in treating respiratory disorders, it has been recognized that use of β-adrenergic agonists can also promote changes outside of the pleural tissue. This has led to widespread adoption of β-adrenergic agonists as repartitioning agents in livestock production (Bergen and Merkel, 1991). Repartitioning agents are compounds that redirect energy away from fat deposition and towards muscle hypertrophy (Ricks et al., 1984). This effect is accomplished by suppressing lipogenic processes (Bergen and Merkel, 1991), in addition to exerting an anabolic effect on protein metabolism in skeletal muscles (Navegantes et al., 2002). When provided to pigs during the finishing period of animal growth, βAAs like ractopamine and cimaterol increase weight gain (Depreux et al., 2002; Carr et al., 2005), promote lean tissue growth (Carr et al., 2005), and improve feed efficiency (Jones et al., 1985) compared with control animals. Additionally, administration of ractopamine to finishing phase pigs increased MyHC-IIB content in the skeletal

muscle while reducing the contribution of other isoforms (Depreux et al., 2002). Although β-adrenergic agonists have been repeatedly shown to improve animal growth performance when used during the finishing phase, it is important to note that animal maturity seems to have a significant influence on the efficacy of β-agonist treatments (Fiems, 1987). For example, a low dose of clenbuterol significantly increased weight gain of lambs weighing 40 kg while it had no effect in lambs weighing 37.5 kg (Baker et al., 1984). Similarly, cimaterol had no effect on carcass leanness when fed to pigs between 10 and 60 kg of body weight (Mersmann et al., 1987) but significantly improved leanness is finishing pigs weighing more than 65 kg (Jones et al., 1985). The reason behind this difference in efficacy has yet to be determined.

In striated muscles, β-agonists promote hypertrophy by increasing protein fractional synthesis rate, translational capacity, and the protein:DNA ratio (Adeola et al., 1992; Nash et al., 1994). An extensive study confirmed that β_2-agonist-induced skeletal muscle hypertrophy is mediated through the AKT-mTOR signaling pathway, where administration of clenbuterol enhanced the phosphorylation of AKT, S6K1, and 4EBP1 in normal weight-bearing rats (Kline et al., 2007). Cross-talk between the adrenergic and AKT-mTOR pathways is facilitated through two mechanism: the first is through direct activation of PI3K by the $G_{\beta\gamma}$ subunits (Kline et al., 2007) and the second through indirect stimulation of AKT activity as a result of cAMP accumulation (Brennesvik et al., 2005). Furthermore, the positive influence of β_2-adrenergic agonists on skeletal muscle hypertrophy is reinforced by suppression of two proteolytic systems: the ubiquitin-proteasome pathway and calpain system. In the same study where Kline et al. linked β_2-agonist use with the AKT-mTOR signaling pathway, they also found that clenbuterol administration could prevent up to 20% of muscle atrophy during hindlimb suspension (Kline et al., 2007). This is likely due to the reduced expression of ubiquitin ligases MuRF1 and Atrogin1 in normal,

denervated, and hindlimb suspension animals dosed with the agonist (Kline et al., 2007). In the calpain system, various β_2-agonists have proven to either downregulate activity of the calpains, enhance the inhibitory activity of calpastatin, or both (Forsberg et al., 1989; Pontremoli et al., 1992; Pringle et al., 1993). It is important to note, however, that stimulation to these pathways by β-agonists is only temporary. The efficacy of β-adrenergic agonists diminishes with increasing exposure due to receptor desensitization (Johnson, 1998; Almeida et al., 2012). Following a process similar to that seen in other G-protein associated systems, receptor desensitization occurs by inactivation through phosphorylation, receptor internalization, and downregulation of adrenoceptor mRNAs (Hausdorff et al., 1990). First, stimulation of the β-adrenergic receptor leads to hyperphosphorylation of the receptor by G-protein coupled receptor kinases (GRKs) (Lohse et al., 1996). This phosphorylation increases the receptor's affinity for members of the arrestin protein family (Lohse et al., 1992). Binding of β-arrestin prevents interaction between the receptor and its associated G-proteins, which restricts any signal transduction following ligand binding (Wilson and Applebury, 1993). In addition, phosphorylation of the receptor and binding of β-arrestin prepares the receptor for internalization (Vasudevan et al., 2011), where the complex is endocytosed and can either be degraded within lysosomes or returned to the cell membrane (Lefkowitz, 1998). Finally, long-term exposure to a β-agonists results in reduced expression of the mRNA coding for the β-adrenergic receptor (Hadcock et al., 1989), a mechanism that is thought to downregulate expression of the receptor at the cell surface.

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Chapter 2. Compensatory Upregulation of Translation Initiation in *Longissimus Dorsi* of Low Birth Weight Pigs at Weaning

ABSTRACT

Low birth weight (LBWT) is associated with impaired perinatal skeletal muscle growth, as well as metabolic dysfunction later in life. We have previously reported a downregulation of protein synthetic pathways in LBWT pigs at birth that may predispose these animals to slower skeletal muscle growth rates compared with their normal birth weight siblings (NBWT). The objectives of this study were to elucidate whether the reduction in protein synthesis observed at birth in LBWT skeletal muscles persists until weaning, and to determine whether protein turnover signaling differs in skeletal muscles with different fiber type compositions. Ten pairs of 1-d old NBWT (1.78 ± 0.06 kg) and LBWT (0.904 ± 0.06 kg) sex-matched littermates (n = 10) were identified at birth. Pigs were allowed to suckle from their sow until 21 d of age, where they were euthanized and muscle and organs collected, weighed, and stored -80°C until analysis. LBWT pigs had consistently smaller absolute weights of organs and skeletal muscles at weaning ($P < 0.05$). While body weight of LBWT pigs was smaller than that of their NBWT siblings both at birth and weaning ($P < 0.0001$), the ratio of LBWT:NBWT weights increased from 50% at birth to 65% at weaning ($P < 0.01$), indicating compensatory growth. mRNA expression of insulin-like growth factor 1 (IGF-1) in the LD was similar for LBWT and NBWT pigs. In addition, the abundance of eukaryotic initiation factor 4E binding protein 1 (4EBP1) and ribosomal protein S6 kinase 1 (S6K1) was similar for both groups. However, phosphorylation of 4EBP1 and eIF4G were greater in skeletal muscle of LBWT compared with NBWT pigs ($P < 0.05$). Although phosphorylation of S6K1 tended to improve, this difference was not significant in LBWT compared with their NBWT siblings. These data suggest that compensatory growth of LBWT pigs between birth and weaning age may be attributed in part to an upregulation of translation initiation signaling in non-oxidative muscles.

INTRODUCTION

Low birth weight (LBWT), which often occurs as a result of intrauterine growth restriction caused by maternal undernutrition, intrauterine crowding, or placental insufficiency, is a major health concern that influences neonatal morbidity and mortality in all mammals (Gaccioli and Lager, 2016; Sharma et al., 2016). The limited nutrient and/or oxygen supplies to the fetus forces a preferential perfusion of key organs at the expense of skeletal muscles (Hernandez-Andrade et al., 2008), causing a disproportionately slow growth and development of prenatal skeletal muscles compared with other tissues (Bauer et al., 1998). This not only decreases the total number of muscle fibers formed during myogenesis (Kuhn et al., 2002; Rehfeldt and Kuhn, 2006), but also results in reduced muscle mass at birth (Powell and Aberle, 1981; Padoan et al., 2004). Neonates afflicted with this condition have the characteristic slow growth rate postnatally (Karlberg and Albertsson-Wikland, 1995), with a distinctive asymmetric growth pattern in which skeletal muscles are most affected (Puiman and Stoll, 2008; Ferenc et al., 2014).

In addition to the slow perinatal growth rate, it is evident that LBWT is correlated with long term consequences, like obesity and metabolic disorders later in life (Barker et al., 2002; Gatford et al., 2010). The current paradigm is that reduced muscle growth in the neonatal period could be a contributing risk for metabolic disorders in adulthood (Brown, 2014). Although "catch-up" growth in commonly occurs in LBWT infants (Karlberg and Albertsson-Wikland, 1995; May et al., 2001), this accelerated weight gain occurs through a simultaneously greater lean tissue and fat accumulation compared with NBWT counterparts and persists into adulthood (Kensara et al., 2005). It is currently not clear what changes occur to protein turnover signaling during the "catch-up" growth period in LBWT neonates.

The early postnatal period is characterized by rapid skeletal muscle hypertrophy (Davis et al., 1993), driven by satellite cell-mediated myonuclear accretion (Bruusgaard et al., 2010) and a greater rate of protein synthesis compared with degradation (Miyazaki 2009). In this regard, we have previously reported that while satellite cell fusion was slightly reduced in LBWT compared with normal birth weight (NBWT) pigs, this reduction is not a major contributor to poor neonatal growth (Chen et al., 2017b). However, we have also reported that downregulated translation initiation in skeletal muscles from LBWT neonatal pigs at birth likely predisposes these animals to slower rates of postnatal muscle growth (El-Kadi et al., 2018). Additionally, these animals have reduced expression of type II myosin heavy chain (MyHC) isoforms, which may correspond to a reduced expression of glycolytic muscle fibers. In the current study, we hypothesized that downregulated translation initiation signaling persists in skeletal muscle of LBWT pigs at weaning, and that this downregulation would be more pronounced in glycolytic compared with oxidative muscles. Our objectives for this study were: to compare protein turnover signaling in LBWT and NBWT pig skeletal muscle at weaning, and to investigate whether protein turnover signaling differs in skeletal muscles with different fiber type compositions.

MATERIALS AND METHODS

Animals and Sample Collection

All procedures involving animals were approved by the Virginia Tech Institutional Animal Care and Use Committee (IACUC). The litters from 8 sows were weighed shortly after birth. Piglets were characterized as normal (NBWT, 1,779 ± 63 g) or low (LBWT, 904 ± 63 g) birth weight when weight at birth was within ± 0.5 SD or ≤ 2 SD below the litter average. Ten pairs of same-sex NBWT-LBWT sibling pairs (n = 10) were identified and remained with their

sow until weaned at 21 d of age. At weaning, all pigs were fasted for 3 hours before euthanasia for sample collection. The longissimus dorsi (LD) and soleus (SOL) muscles, in addition to the liver, heart, and kidney, were dissected from the carcass and weighed before snap-freezing in liquid nitrogen. All tissue samples were then stored at -80°C for further analysis.

Total RNA and Protein Quantification

To determine total RNA and protein concentration, 100 mg of frozen muscle was homogenized in 0.2 M perchloric acid (PCA). Following two washes with additional 0.2 M PCA, the pellet was resuspended with 0.3 M sodium hydroxide and incubated at 37°C for 1 hr. Total protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Wilmington, DE). Proteins were then reprecipitated with 2 M PCA and incubated on ice for 10 mins. After centrifugation, total RNA concentration of the supernatant was quantified spectrophotometrically (NanoDrop, Thermo Fisher Scientific, Wilmington, DE).

RNA Extraction and Quantitative Real-Time PCR

Skeletal muscle total RNA was extracted from approximately 50 mg of LD or SOL tissue using the Direct-zol RNA Miniprep Kit (ZYMO Research, Orange, CA). The resulting concentration of total RNA was then quantified spectrophotometrically (NanoDrop, Thermo Fisher Scientific, Wilmington, DE) and diluted to a concentration of 200 ng/µl, followed by reverse transcription into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Gene-specific primers (Table 2-1) and Fast SYBR Green Master Mix (Applied Biosystems) were mixed with cDNA samples and plated in triplicates into 96-well plates. Real-time quantitative PCR (qPCR) reactions were performed on an ABI 7500 Fast Real-time PCR cycler (Applied Biosystems). Relative mRNA expression was calculated using the 2^{-}

$^{\Delta\Delta CT}$ method with 18S abundance used for normalization. All primer sets were evaluated for adequate efficiency and validated by Sanger sequencing before use in this study.

Protein Abundance and Phosphorylation

Muscle lysates were analyzed by western blotting to determine protein abundance and phosphorylation. Approximately 100 mg of LD or SOL muscle tissue was homogenized in a homogenization buffer (1:7 w/v), containing 100 mM potassium chloride, 50 mM β-glycerophosphate, 50 mM sodium fluoride, 20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 2 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 0.2 mM ethylenediaminetetraacetic acid (EDTA), and protease inhibitor cocktail (1:150; P8340, Sigma, St. Louis, MO). Tissue lysate samples were then diluted (1:1) in 2× Laemmli Sample Buffer (Bio-Rad, Hercules, CA) and boiled at 100°C for 5 min.

Equal volumes of prepared samples were separated by electrophoresis through 4-12% polyacrylamide gels. Following separation, proteins were then electrophoretically transferred to a polyvinylidene difluoride membrane (Thermo Scientific). Membranes were blocked for 2 h at room temperature with 3% bovine serum albumin (Research Products International Corp, Mount Prospect, IL) in tris-buffered saline with 0.1% Tween-20 (TBST) to reduce non-specific binding to the membrane. Incubation with protein-specific primary antibodies (Table 2) was performed overnight at 4°C with agitation. The blots were extensively washed in TBST before incubation with horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse or goat anti-rabbit IgG-HRP conjugate; Bio-Rad) for 1 h at room temperature. The blots were again washed in TBST and then imaged for chemiluminescence using the Novex™ ECL Chemiluminescent Substrate Reagent Kit (Thermo Fisher Scientific) and a BioDoc digital imager (Bio-Rad). Band optical

density was obtained using Image Lab 4.0 software (Bio-Rad). Total intensity values were normalized against α-tubulin as an internal loading control.

Quantification of eIF4E Associations

To quantify the association of eIF4E with 4EBP1 and eIF4G, tissue homogenates were further processed by immunoprecipitation. For the eIF4E:4EBP1 complex, lysates were incubated with an anti-eIF4E antibody (Table 2-2). The resulting immunocomplexes were precipitated with goat anti-rabbit IgG magnetic beads (Polysciences, Warrington, PA), followed by vigorous washing with phosphate-buffered saline (PBS). Captured immunoprecipitates were resuspended in 1× Laemmli Sample Buffer (Bio-Rad) and boiled at 100°C for 5 min. Once centrifuged at 16,000 × g for 5 mins at 4°C, the supernatants were collected for SDS-PAGE, followed by immunoblotting with anti-eIF4E and anti-4EBP1 antibodies. To quantify the interaction of eIF4E with eIF4G, a similar technique was employed where tissue lysates were incubated with an anti-eIF4G antibody and then prepared for immunoblotting with anti-eIF4E and anti-eIF4G antibodies.

Statistical Analysis

Data were analyzed using PROC MIXED in SAS version 9.3 (SAS Institute Inc., Cary, NC). Birth weight was used as the main effect while sow number and sex were treated as random effects. Data are presented as the least square means ± standard error. Differences are considered significant at $P \leq 0.05$, unless otherwise noted.

RESULTS

Body and Tissue Weights

Both the birth weights (Fig. 2-1A) and body weights at weaning (Fig. 2-1B) were significantly lower for LBWT compared to NBWT piglets ($P < 0.0001$). When body weights of

sibling pairs were represented as a ratio of LBWT:NBWT (Fig. 2-1C), LBWT pigs are approximately half the weight of their NBWT siblings as neonates and only ~35% lighter at weaning ($P < 0.01$). The absolute weights of the heart, liver, and kidney (Table 2-3) from LBWT pigs weighed 30%, 30%, and 32% less than those from NBWT pigs, respectively ($P \leq 0.01$). Similarly, the absolute weights of the longissimus dorsi (LD) and soleus (SOL) muscles (Table 2-3) from LBWT pigs were 38% and 42% lighter ($P \leq 0.001$). Expressed as a percentage of body weight, the weights of the heart, liver, and kidney (Table 2-3) were not significantly different between groups. For the skeletal muscles, the SOL accounted for a significantly smaller proportion of total body weight in LBWT pigs compared with NBWT siblings ($P < 0.05$; Table 2-3), while the weight of the LD as a percent of body weight was not significantly different between birth weight classes (Table 2-3).

RNA Content and Translational Capacity

The total RNA content and total protein content of muscles samples were used to estimate translational capacity. Birth weight had no effect on the RNA content, protein content, or translational capacity (Fig. 2-2A-C) in either the LD or SOL muscle. In contrast, the RNA content and translational capacity were greater in the SOL compared with the LD muscle.

Expression of Myosin Heavy Chain

We evaluated the mRNA expression of MHC7, MHC2, MHC1, and MHC4, which correspond to myosin heavy chain (MyHC) isoforms MyHC I, MyHC IIA, MyHC IIX, and MyHC IIB, respectively. In the LD, the expression of MyHC genes were not different between LBWT and NBWT pigs (Fig. 2-3A-D). Similarly, there was no difference between expression of MyHC I, IIA, and IIB between LBWT and NBWT pigs in the soleus muscle (Fig. 2-4A, B, and D).

However, the expression of MyHC IIX was found to be significantly elevated in the SOL muscle of LBWT pigs (Fig. 2-4C).

IGF System and MSTN Signaling

The expression of IGF-1 or IGF-2 mRNA was not different between LBWT and NBWT pigs for either the LD (Fig. 2-5A and B) or the SOL (Fig. 2-7A and B) muscles. Similarly, birth weight did not affect the expression of IGF-IR in either muscle (Fig. 2-5C and 2-6C). While there was no difference in the expression of MSTN (Fig. 2-7A and 2-8A) between LBWT and NBWT pigs in either the LD or SOL muscles, we found greater expression of DCN (Fig. 2-8B) in the SOL of LBWT pigs ($P > 0.01$).

Translation Initiation Signaling

In the LD muscle, we found no difference in the total protein abundance of eIF4E. Similarly, the abundance of 4EBP1, eIF4G, or S6K1 in LD muscle lysates were not found to be different between the birth weight groups (Fig. 2-9A-C). However, there was a significant upregulation in the phosphorylation of both 4EBP1 and eIF4G in LBWT pigs ($P < 0.05$) (Fig. 2-9D-E). While not significant, the phosphorylation of S6K1 (Fig. 2-9F) was numerically higher in LBWT pigs ($P = 0.09$). Additionally, there was no difference in the association of eIF4E with either 4EBP1 or eIF4G (Fig. 2-11A-C) between LBWT and NBWT pigs when analyzed by immunoprecipitation.

Similar to our findings in the LD muscle, the total abundance of eIF4E, 4EBP1, or S6K1 in the SOL were unaffected by birth weight. However, the total abundance of eIF4G tended to be slightly elevated in the SOL muscle of LBWT pigs ($P = 0.051$) (Fig. 2-10B). Additionally, we found no differences in the phosphorylation status of either 4EBP1, eIF4G, or S6K1 (Fig. 2-10D-

F). There was no difference in the association of eIF4E with either 4EBP1 or eIF4G (Fig. 2-12A-C) between LBWT and NBWT pigs when analyzed by immunoprecipitation.

Protein Degradation Signaling

From the calcium-dependent protease system, we found no difference in the protein abundance of Calpain-1 or Calpain-2 in the LD of LBWT and NBWT pigs (P = 0.081 and 0.173, respectively) (Fig. 2-13C and D). Birth weight did not significantly alter the abundance of either calpain protein in the SOL muscle (P = 0.340 and 0.895, respectively) (Fig. 2-14C and D).

We also evaluated the abundance of the skeletal muscle-specific E3 ligases, muscle atrophy F-Box protein (Atrogin-1) and muscle RING finger protein 1 (MuRF1), from the ubiquitin-proteasome pathway. MuRF1 abundance in the LD of LBWT pigs tended to be reduced compared with NBWT pigs (P = 0.062) (Fig. 2-13B). The abundance of Atrogin-1 in the LD (P = 0.533), as well as abundance of both Atrogin-1 and MuRF1 in the SOL (P = 0.590 and 0.340, respectively), were not found to be different between LBWT and NBWT pigs (Fig. 2-13A, 2-14A and B).

DISCUSSION

Compensatory growth of LBWT neonates is common during early postnatal life (Druet and Ong, 2008), and is thought to reduce neonatal morbidity and improved cognitive function (Lundgren et al., 2001; Victora et al., 2001; Radlowski et al., 2014). However, LBWT infants that undergo this compensatory growth are also more likely to develop hypertension, heart disease, and metabolic dysfunction in adulthood (Jaquet et al., 2000; Eriksson et al., 2001; Barker et al., 2002). Though their accelerated weight gain is often attributed to increased fat deposition (Ibanez et al., 2011; Cho and Suh, 2016), little is known about the changes to protein turnover signaling in skeletal muscles that occur as LBWT neonates experience compensatory growth. In the current

study, the neonatal piglet was used as a model for human infants due to their physiologic and metabolic similarities in the early postnatal period (Puiman and Stoll, 2008; Ferenc et al., 2014). We have previously reported that translation initiation is downregulated in neonatal LBWT pigs compared with their NBWT littermates, and that those differences were associated with a reduction in type II MyHC expression (Chen et al., 2017a; El-Kadi et al., 2018). In light of these findings, we sought to determine whether the downregulation of translation initiation signaling pathways reported in LBWT pigs at birth persists until weaning at 21 days of age, and to elucidate whether differences in protein turnover signaling that occur as a result of LBWT are correlated with changes in fiber types.

As expected, body weight was greater for NBWT pigs compared with their LBWT siblings at birth and at weaning. Despite this persistent difference, the weight of LBWT pigs as a percent of their NBWT counterparts increased from 50% at birth to 65% at weaning, suggesting that LBWT pigs experienced limited compensatory growth during the suckling period. These data are consistent with observations in infants (Hack et al., 1996; Beger et al., 2018) and pigs (Handel and Stickland, 1988; Ritacco et al., 1997) that LBWT neonates commonly undergo compensatory growth in early postnatal life. Although intake of breast milk or formula was not measured, it has been suggested that compensatory growth of LBWT infants may be attributed greater appetite (Martos-Moreno et al., 2009; Fidancı et al., 2010). While a limitation of the current study was that pigs were sow-raised and milk intake was not measured, we consider it unlikely that LBWT pigs would have greater nutrient intake compared with their larger littermates. For instance, voluntary milk intake of LBWT infants is less than their NBWT counterparts despite achieving greater daily weight gain (Ounsted and Sleigh, 1975). Similarly, while voluntary formula intake of LBWT pigs

during the early postnatal period was similar to their NBWT counterparts, LBWT pigs experienced compensatory growth (Myrie et al., 2017).

Weight gain could occur as a result of greater lean or fat deposition. This is why two skeletal muscles were used to assess lean tissue deposition: the longissimus dorsi (LD) and the soleus (SOL) muscles. Our data indicated that LD and SOL muscle weights were less in LBWT pigs compared with their NBWT siblings. These findings are consistent with previous reports indicating that skeletal muscle weight is less in LBWT neonatal pigs compared with their NBWT counterparts (Kuhn et al., 2002; Wang et al., 2008; El-Kadi et al., 2018). Similarly, reduced muscularity, measured via mid-upper arm anthropometry, has been reported in small-for-gestational-age infants and young children compared with those born large-for-gestational-age (Hediger et al., 1998). Evidently, musculature would be expected to grow proportionately to the rest of the body. However, when muscle mass was expressed as a percentage of body weight, only the SOL from LBWT pigs represented a smaller proportion of body weight compared with those from NBWT pigs. These data suggest that accelerated hypertrophy of some muscles contributes in part to the compensatory growth of LBWT pigs. Although we have previously reported that LD weight relative to whole body weight was greater for NBWT pigs compared with their LBWT siblings at birth (El-Kadi et al., 2018), in the current study, the disparity between growth of the LD and SOL of LBWT compared with NBWT pigs likely reflects fiber type differences between the two muscles studied.

In pigs, myogenesis occurs in two phase: primary between 35 and 55 days and secondary between 55 and 95 of gestation (Wigmore and Stickland, 1983). The many of the secondary fiber population mature into glycolytic fibers in postnatal muscles (Picard et al., 2002). It is well established that intrauterine growth restriction hinders secondary myogenesis, thereby reducing

total fiber numbers and limiting secondary fiber formation (Wigmore and Stickland, 1983; Kuhn et al., 2002) while primary muscle fibers are generally unaffected (Aberle, 1984). In this regard, the LD is a predominantly glycolytic fast-twitch muscle whereas the SOL is a highly oxidative slow-twitch muscle. We have previously reported a reduction in for the myosin heavy chain (MyHC) mRNA expression of glycolytic fibers (i.e. MyHC IIX and IIB) in the LD of LBWT pigs at birth (El-Kadi et al., 2018). In the current study, however, expression of MyHC isoforms in the LD and SOL (except for MyHC IIX) was similar for LBWT and NBWT pigs, indicating a shift in fiber type composition towards a more glycolytic phenotype. Fractional synthesis and degradation rates are greater in oxidative muscles compared with glycolytic muscles in many species including rats (Davis et al., 1989; Garlick et al., 1989), chickens (Laurent et al., 1978), and pigs (Chang and Wei, 2005). The higher rate of protein turnover in oxidative muscles would suggest that they are less efficient than glycolytic muscles at depositing energy and amino acids into tissue protein (van Wessel et al., 2010).

Protein turnover is a highly regulated process that determines the extent of skeletal muscle protein accretion or loss. The rate of protein synthesis is enhanced through an increase in translational capacity or efficiency (Bush et al., 2003). Since 80-85% of the cellular RNA pool is ribosomal (Henshaw et al., 1971), the ratio of RNA to total protein content is often used as surrogate measure of ribosomal abundance, and thus translational capacity (Fiorotto et al., 2014). In the current study, translational capacity was greater for the SOL than the LD, whereas capacity was similar between birth weight groups in either the LD or SOL muscles. Previous work in rat muscle has reported oxidative fibers have greater total RNA expression than glycolytic fibers (Habets et al., 1999). This should correlate with a higher rate of protein synthesis due to greater availability of translational machinery. However, as previous mentioned, the rate of protein

degradation is greater in oxidative muscles (Laurent et al., 1978; Davis et al., 1989; Chang and Wei, 2005), which likely contributes to the difference in hypertrophic potential between glycolytic and oxidative muscles. Additionally, considering that translational capacity was similar in the LD of LBWT and NBWT pigs, this would suggest the compensatory growth was related to an enhanced efficiency of protein translation via alterations in its regulatory mechanisms.

Translation initiation is the rate-limiting step of protein synthesis. We have previously reported downregulation of translation initiation signaling in LD of LBWT pigs compared to their NBWT siblings at birth (Chen et al., 2017a; El-Kadi et al., 2018). For example, there was a reduction in S6K1 phosphorylation, and abundance and phosphorylation of eIF4G in the LD of LBWT neonatal pigs compared with their NBWT siblings (Chen et al., 2017a). In the current study, despite greater phosphorylation of 4E-BP1 and eIF4G in the LD and greater eIF4G abundance in the SOL, eIF4E:eIF4G complex formation was similar for both muscles of LBWT and NBWT pigs. Although to account for compensatory growth we expected that translation initiation signaling be enhanced or that protein degradation be reduced, the current data suggest that downregulated translation initiation signaling reported for LBWT pigs at birth compared with their NBWT siblings (Chen et al., 2017a; El-Kadi et al., 2018) returned to normal by 21 d. It is likely that translation initiation was enhanced prior to termination of the study.

Another major point of regulation for the IGF-1-PI3K-AKT-mTORC1 pathway is through the myostatin signaling pathway. Myostatin (MSTN), part of the transforming growth factor-β (TGF-β) superfamily, is a potent inhibitor of skeletal muscle growth (Miura et al., 2006). When not bound in an inhibitory complex with proteoglycan decorin (DCN), MSTN blocks activation of AKT, thus inhibits stimulation of protein synthesis (Miura et al., 2006). We have previously reported that the MSTN signaling pathway was downregulated through a reduction in MSTN and

greater DCN expression in the LD muscle of LBWT piglets (Chen et al., 2017a), suggesting that this downregulation was compensatory in nature. In the current study, there were no differences in the expression of MSTN in either the LD or SOL muscle between LBWT and NBWT pigs at weaning. While birth weight had no effect on expression of DCN in the LD, DCN expression was ~65% greater in the SOL of LBWT pigs compared with those from NBWT siblings. Although an upregulation of DCN in the SOL would promote hypertrophy by inhibiting MSTN there was no effect on muscle weigh of the SOL. This, in combination with the loss of downregulation to the MSTN pathway that was once seen in the neonatal LBWT LD (Chen et al., 2017a), support our previous findings that changes to the MSTN signaling pathway in LBWT pigs represent a compensatory mechanism.

In conclusion, our data suggest that LBWT pig experienced compensatory growth by 21 days of age. Our data also suggest that this accelerated growth compensatory growth occurred in in glycolytic muscles like the LD, whereas this growth was less pronounced in oxidative muscles like the soleus. Finally, although translation initiation and protein degradation signaling were similar in the skeletal muscle of LBWT and NBWT pigs, it is plausible that changes in these pathways may have occurred prior to termination of the study.

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Table 2-1. Nucleotide sequences for real-time PCR primers

Target	Direction	Primer Sequence	Accession No.
18 S	Forward	5'- GTA ACC CGT TGA ACC CCA T -3'	AY265350
	Reverse	5'- CCA TCC AAT CGG TAG TAG CG -3'	
Decorin	Forward	5'- ATC TCA GCT TTG AGG GCT CC -3'	NM_213920.1
	Reverse	5'- TGT CCA GAC CCA AAT CAG AAC AT -3'	
IGF-I	Forward	5'- GCA CAT CAC ATC CTC TTC GC -3'	NM_214256.1
	Reverse	5'- ACC CTG TGG GCT TGT TGA AA -3'	
IGF-I receptor	Forward	5'- CAT ACC AGG GCT TGT CCA AC -3'	NM_214172.1
	Reverse	5'- ATC AGC TCA AAC AGC ATG TCG -3'	
Myosin heavy chain I	Forward	5'- CTG TCC AAG TTC CGC AAG GT -3'	NM_213855.2
	Reverse	5'- TCC TCG TTC AAG CCC TTG GT -3'	
Myosin heavy chain IIA	Forward	5'- CTC ACT TGC TAA GAA GGA CCT CTG -3'	NM_214136.1
	Reverse	5'- GCC TCA ATG CGC TCC TTT TC -3'	
Myosin heavy chain IIB	Forward	5'- GTA CAT CTA GTG CCC TGC TGC -3'	NM_001123141.1
	Reverse	5'- CCC TGC TTC CGT CTT CAC TG -3'	
Myosin heavy chain IIX	Forward	5'- ACA GGA ACA CCC AAG GCA TC -3'	NM_001104951.2
	Reverse	5'- GAG CTC CTG TTC TGC GAC TT -3'	
Myostatin	Forward	5'- CCA GAG AGA TGA CAG CAG TGA TG -3'	NM_214435.2
	Reverse	5'- TTC CTT CCA CTT GCA TTA GAA GAT C -3'	

Table 2-2. Primary antibodies used for western blotting

Antibody	Company
4EBP1	#A300-501A; Bethyl Laboratories, Montgomery, TX
Atrogin-1	#AP2041; ECM Biosciences, Versailles, KY
Calpain 1	#2556; Cell Signaling Technology, Danvers, MA
Calpain 2	#373967; Santa Cruz Biotechnology, Dallas, TX
eIF4AI/II	#sc-377315; Santa Cruz Biotechnology, Dallas, TX
eIF4B	#sc-376062; Santa Cruz Biotechnology, Dallas, TX
eIF4E	#9742; Cell Signaling Technology, Danvers, MA
eIF4G	#8701; Cell Signaling Technology, Danvers, MA
IGF-I receptor β	#9750; Cell Signaling Technology, Danvers, MA
MuRF1	#AF5366; R&D systems, Minneapolis, MN
p70 S6 kinase	#2708; Cell Signaling Technology, Danvers, MA
phospho-4EBP1 (Thr46)	#44-1170G; Invitrogen, Camarillo, CA
phospho-eIF4E (Ser209)	#9741; Cell Signaling Technology, Danvers, MA
phosphor-eIF4G (Ser1108)	#2441; Cell Signaling Technology, Danvers, MA
phospho-S6K1 (Thr389)	#07-018-I; Millipore, Temecula, CA
α-Tubulin	#H300; Santa Cruz Biotechnology, Dallas, TX

Table 2-3. Body and organ weights

	Birth Weight			
	NBWT	LBWT	SEM	*P*-Value
Birth weight, *kg*	1.8	0.9	0.063	< **0.0001**
Weaning weight, *kg*	6.5	4.3	0.643	< **0.0001**
Absolute Weight, *g*				
Longissimus dorsi	107.9	66.8	16.324	< **0.001**
Soleus	5.0	2.9	0.721	< **0.0001**
Liver	169.5	117.9	19.429	< **0.001**
Heart	38.6	26.9	2.260	< **0.01**
Kidney	17.9	12.2	0.883	< **0.001**
Percentage of Body Weight, *%*				
Longissimus dorsi	1.8	1.5	0.096	0.085
Soleus	0.08	0.07	0.006	< **0.05**
Liver	2.6	2.8	0.1144	0.381
Heart	0.6	0.6	0.023	0.289
Kidney	0.3	0.3	0.013	0.434

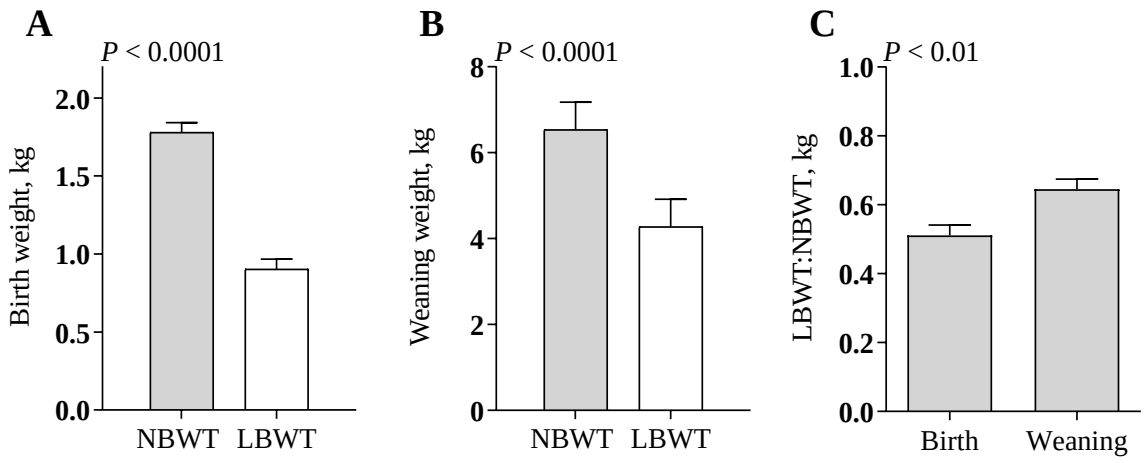

Figure 2-1. Birth weight (A), body weight at weaning (B), and ratio of LBWT:NBWT body weight over experimental period for low birth weight (LBWT) and normal birth weight (NBWT) pigs. Results are means ± SE.

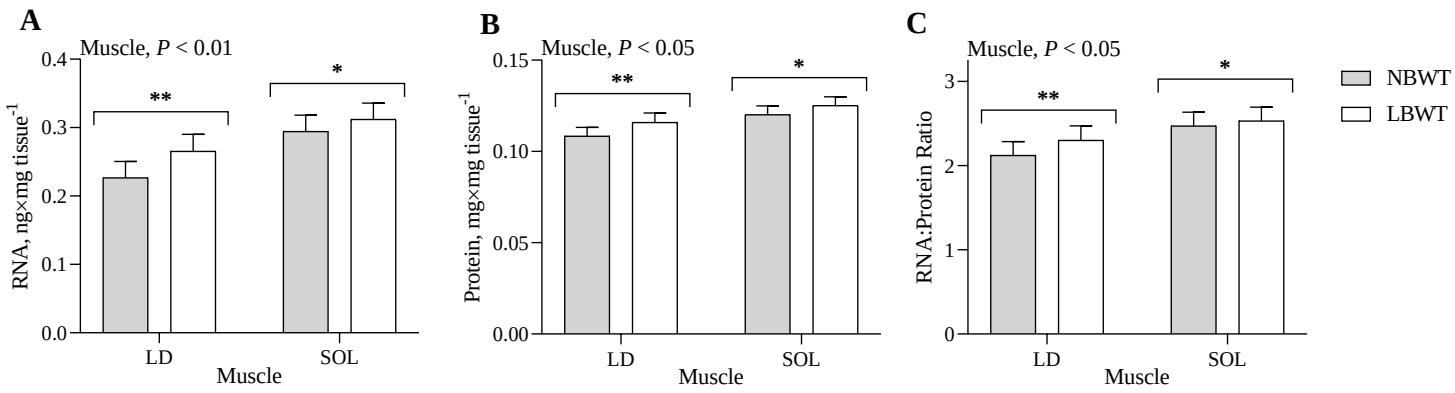

Figure 2-2. RNA content (A), protein content (B), and RNA:protein ratio (C) in the longissimus dorsi and soleus of low birth weight (LBWT) and normal birth weight (NBWT) pigs. Results are means ± SE.

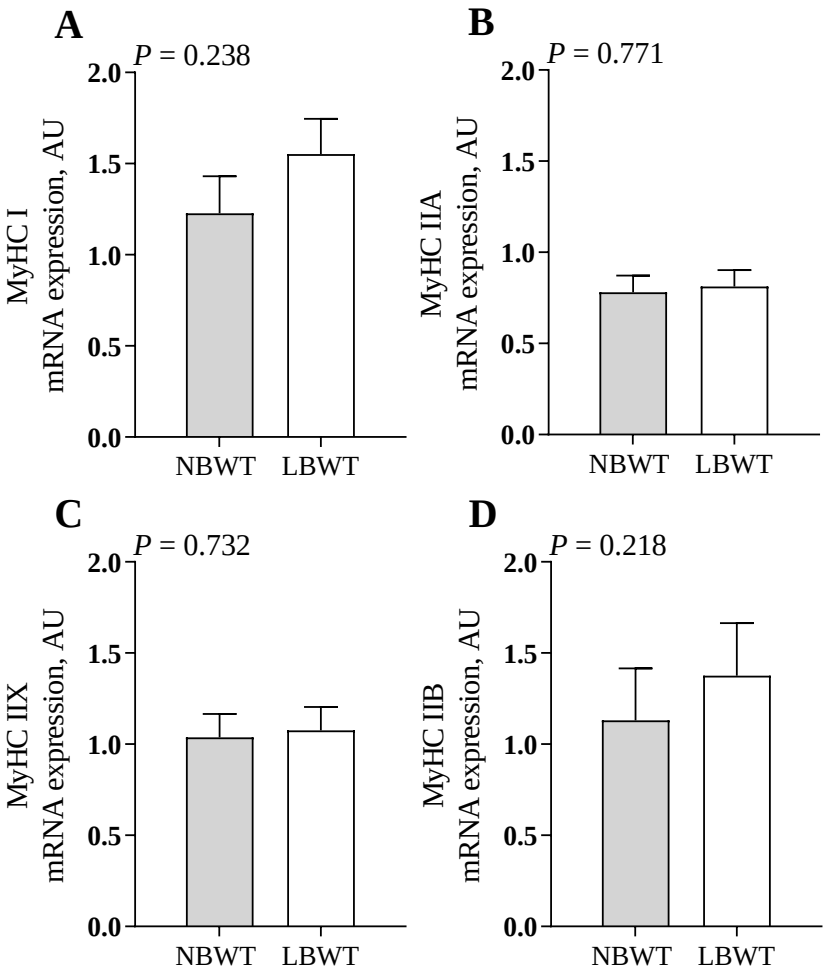

Figure 2-3. mRNA expression of the myosin heavy chain (MyHC) isoforms in the longissimus dorsi muscle of low birth weight (LBWT) and normal birth weight (NBWT) pigs. Results are means ± SE.

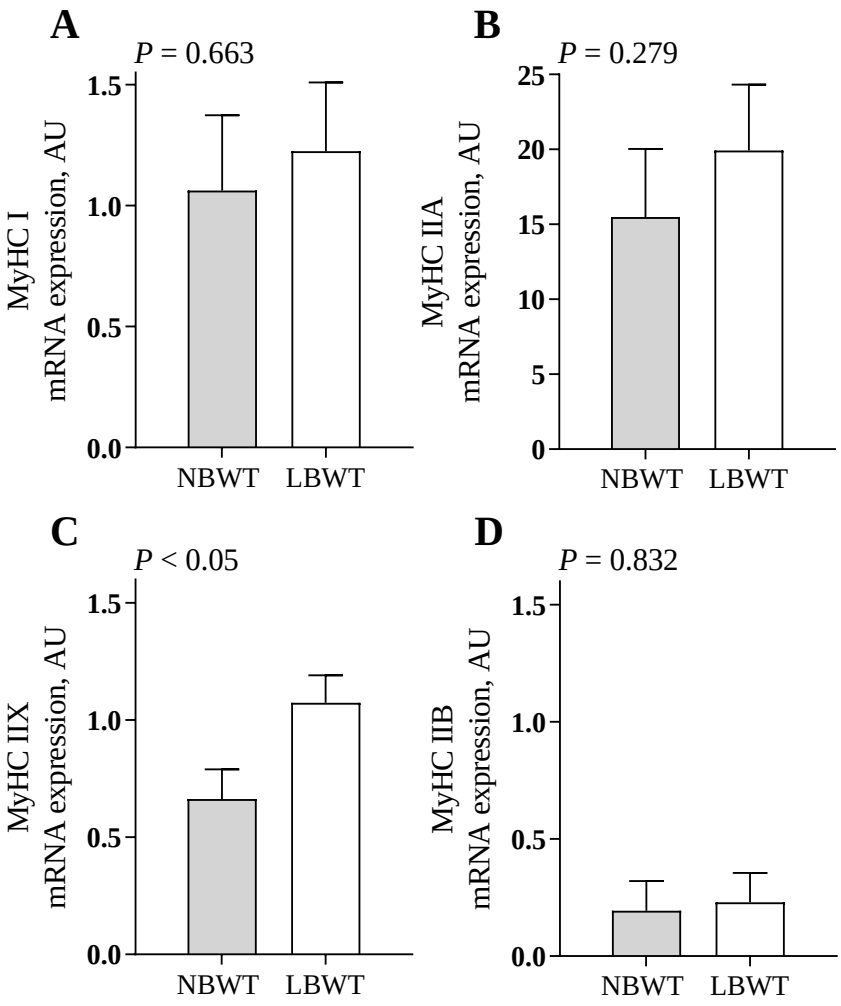

Figure 2-4. mRNA expression of the myosin heavy chain (MyHC) isoforms in the soleus muscle of low birth weight (LBWT) and normal birth weight (NBWT) pigs. Results are means ± SE.

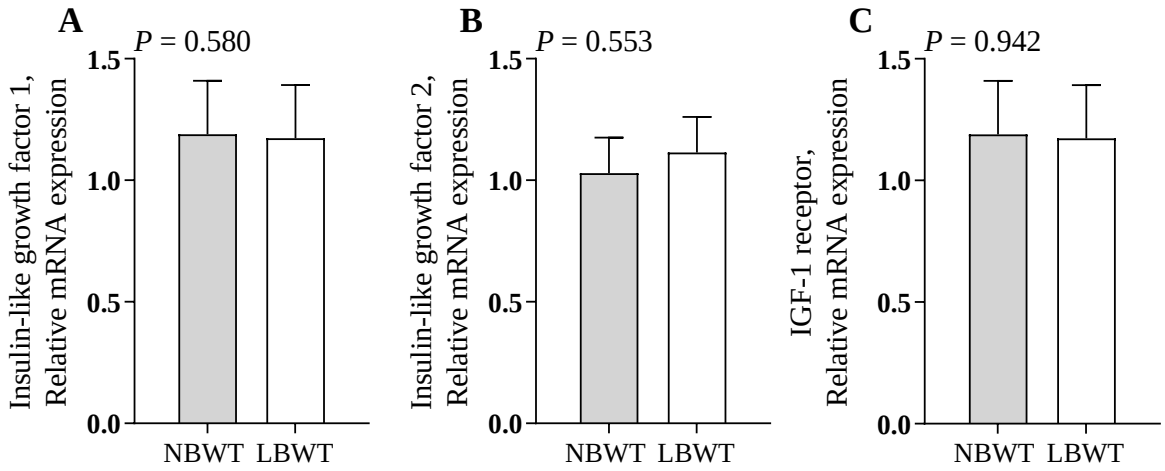

Figure 2-5. mRNA expression of insulin-like growth factor 1 (IGF-1) (A), insulin-like growth factor 2 (IGF-2) (B), and insulin-like growth factor 1 receptor (IGF-IR) (C) in the longissimus dorsi muscle of low birth weight (LBWT) and normal birth weight (NBWT) pigs. Results are means ± SE.

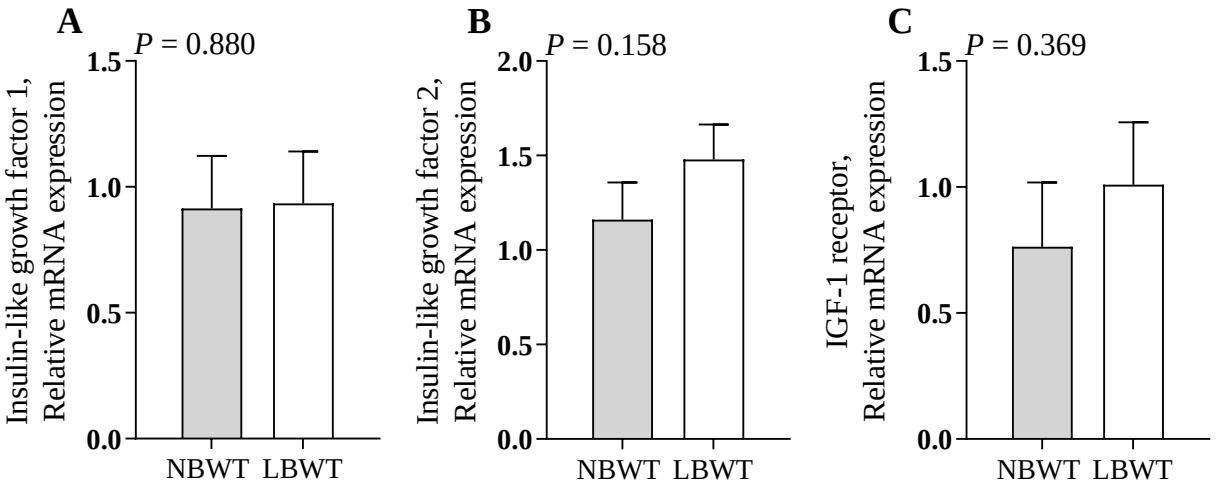

Figure 2-6. mRNA expression of insulin-like growth factor 1 (IGF-1) (A), insulin-like growth factor 2 (IGF-2) (B), and insulin-like growth factor 1 receptor (IGF-IR) (C) in the soleus muscle of low birth weight (LBWT) and normal birth weight (NBWT) pigs. Results are means ± SE.

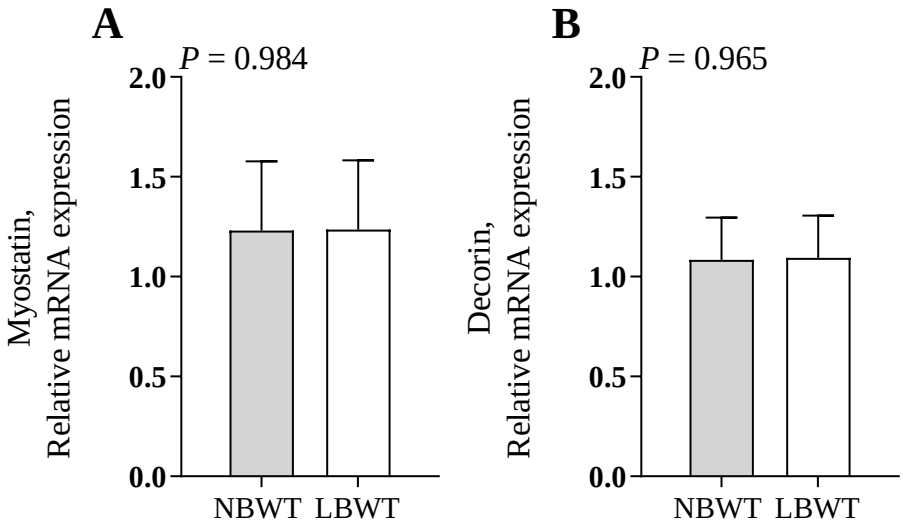

Figure 2-7. mRNA expression of myostatin (A) and decorin (B) in the longissimus dorsi muscle of low birth weight (LBWT) and normal birth weight (NBWT) pigs.

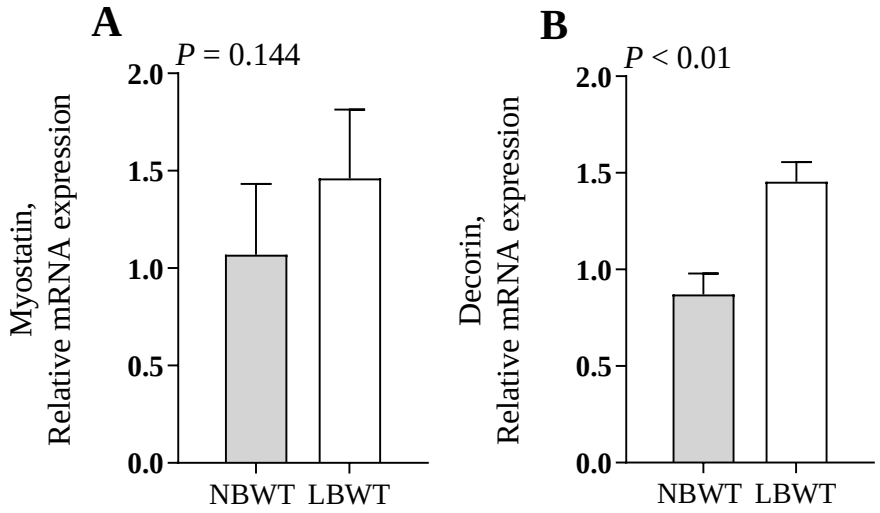

Figure 2-8. mRNA expression of myostatin (A) and decorin (B) in the soleus muscle of low birth weight (LBWT) and normal birth weight (NBWT) pigs. Asterisks indicate significant difference ($P \leq 0.05$). Results are means ± SE.

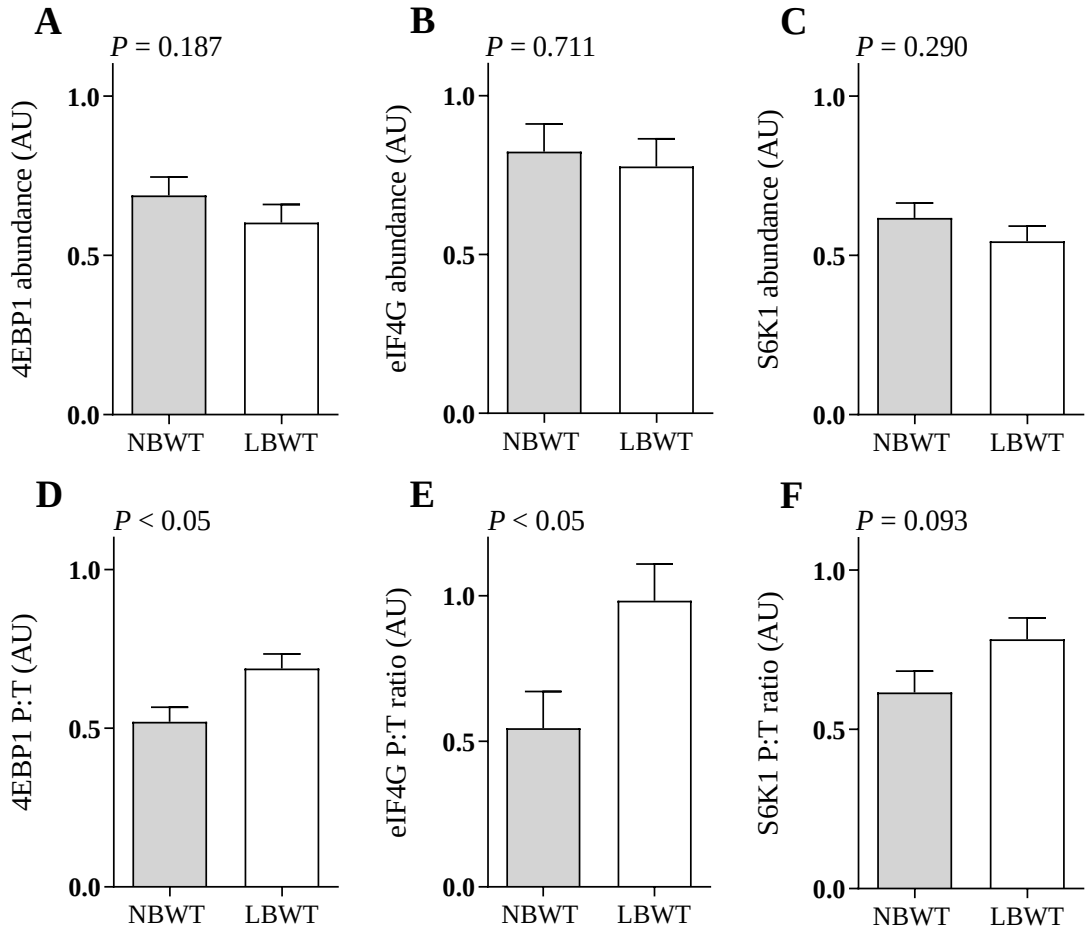

Figure 2-9. Total protein abundance of Eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) (A), Eukaryotic translation initiation factor 4G (eIF4G) (B), and ribosomal protein S6 kinase 1 (S6K1) (C), phoshorylation of 4EBP1 (D), phosphorylation of eIF4G (E), and phosphorylation of S6K1 (F) in the longissimus dorsi muscle of low birth weight (LBWT) and normal birth weight (NBWT) pigs. Results are means ± SE.

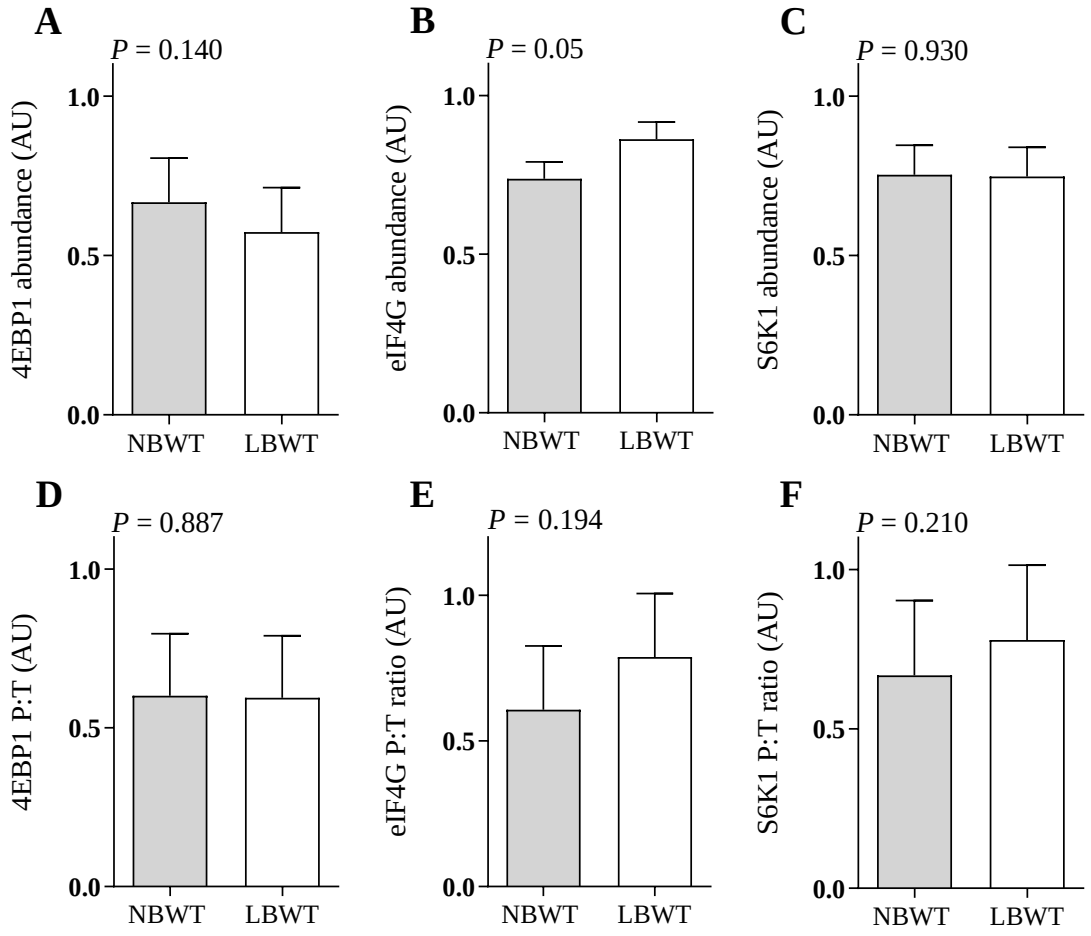

Figure 2-10. Total protein abundance of Eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) (A), Eukaryotic translation initiation factor 4G (eIF4G) (B), and ribosomal protein S6 kinase 1 (S6K1) (C), phoshorylation of 4EBP1 (D), phosphorylation of eIF4G (E), and phosphorylation of S6K1 (F) in the soleus muscle of low birth weight (LBWT) and normal birth weight (NBWT) pigs. Results are means ± SE.

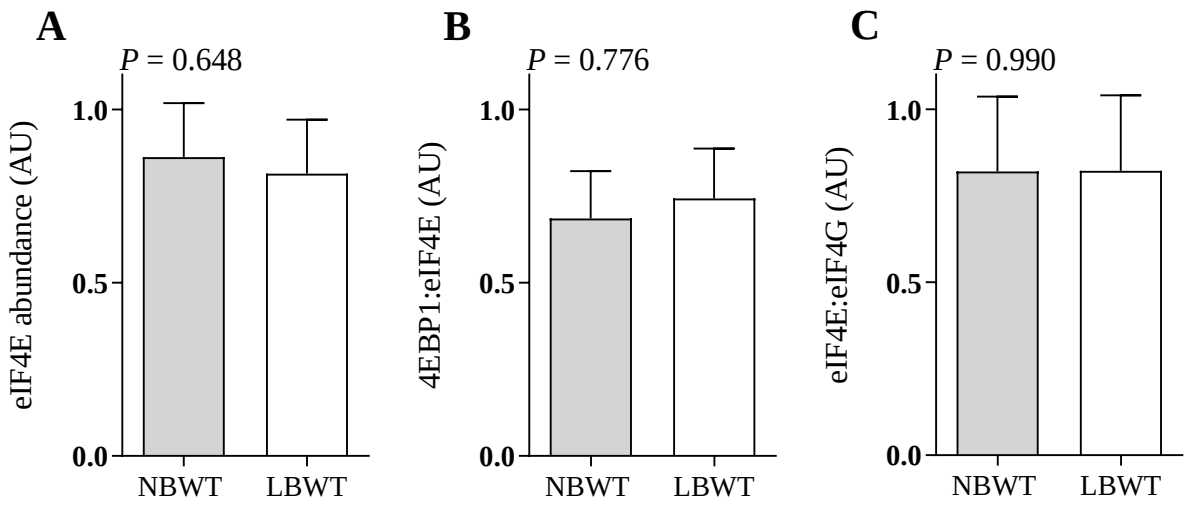

Figure 2-11. Total abundance of Eukaryotic initiation factor 4E (eIF4E) (A), eIF4E-4EBP1 complex (B), and eIF4E-eIF4G complex (C) in the longissimus dorsi of low birth weight (LBWT) and normal birth weight (NBWT) pigs. Results are means ± SE.

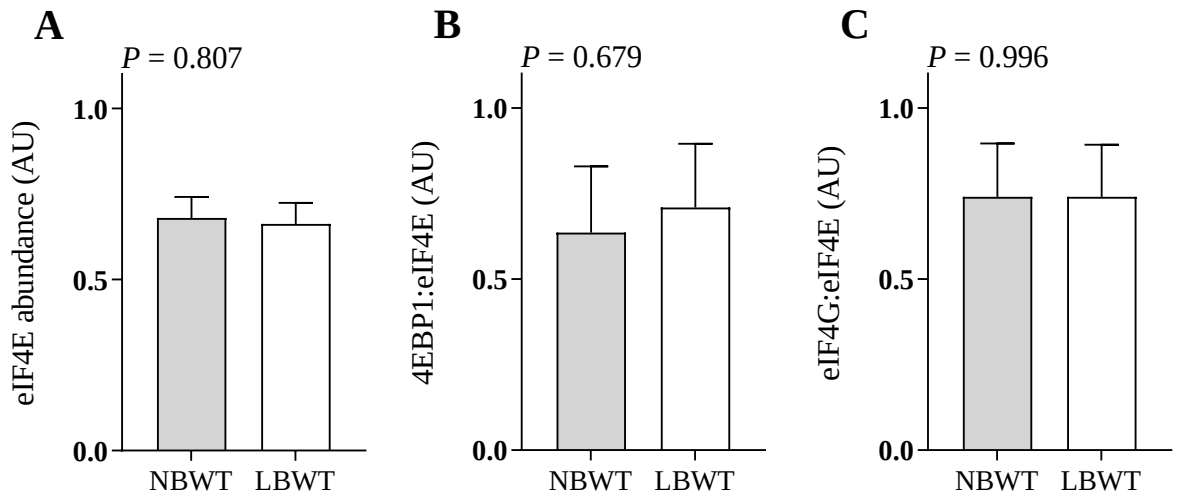

Figure 2-12. Total abundance of Eukaryotic initiation factor 4E (eIF4E) (A), eIF4E-4EBP1 complex (B), and eIF4E-eIF4G complex (C) in the soleus of low birth weight (LBWT) and normal birth weight (NBWT) pigs. Results are means ± SE.

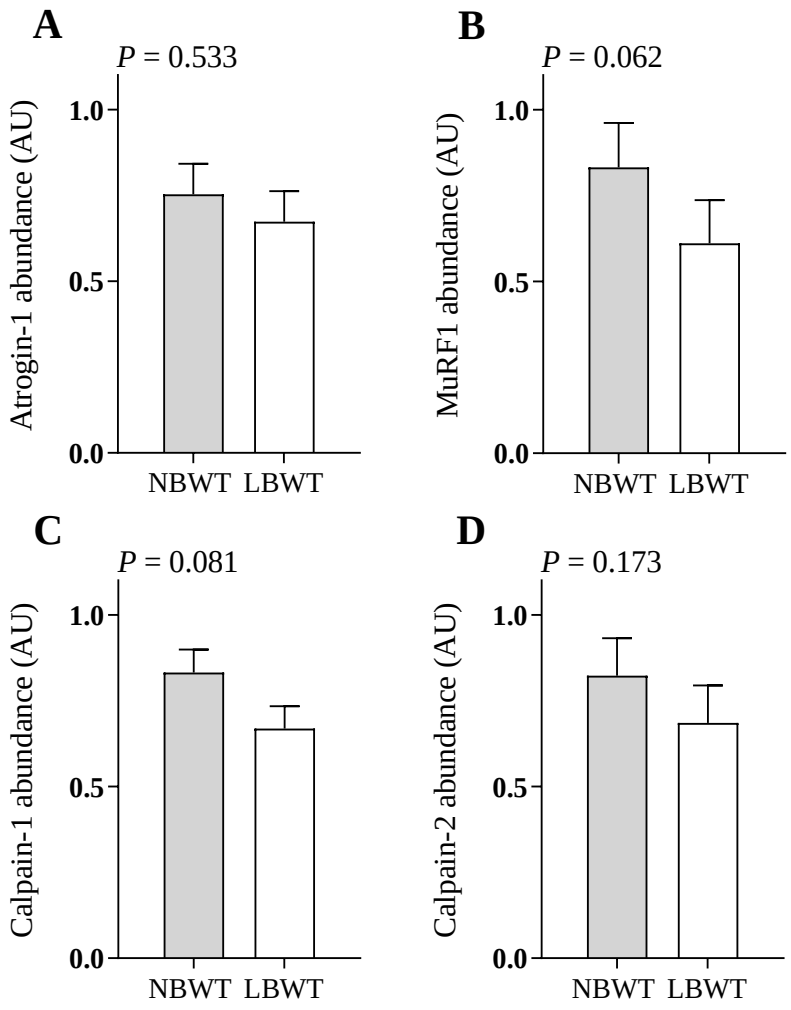

Figure 2-13. Total abundance of muscle-specific E3 ubiquitin ligases, Atrogin-1 (A) and MuRF1 (B), as well as calcium-dependent proteases, Calpain-1 (C), and Calpain-2 (D), in the longissimus dorsi of low birth weight (LBWT) and normal birth weight (NBWT) pigs. Results are means ± SE.

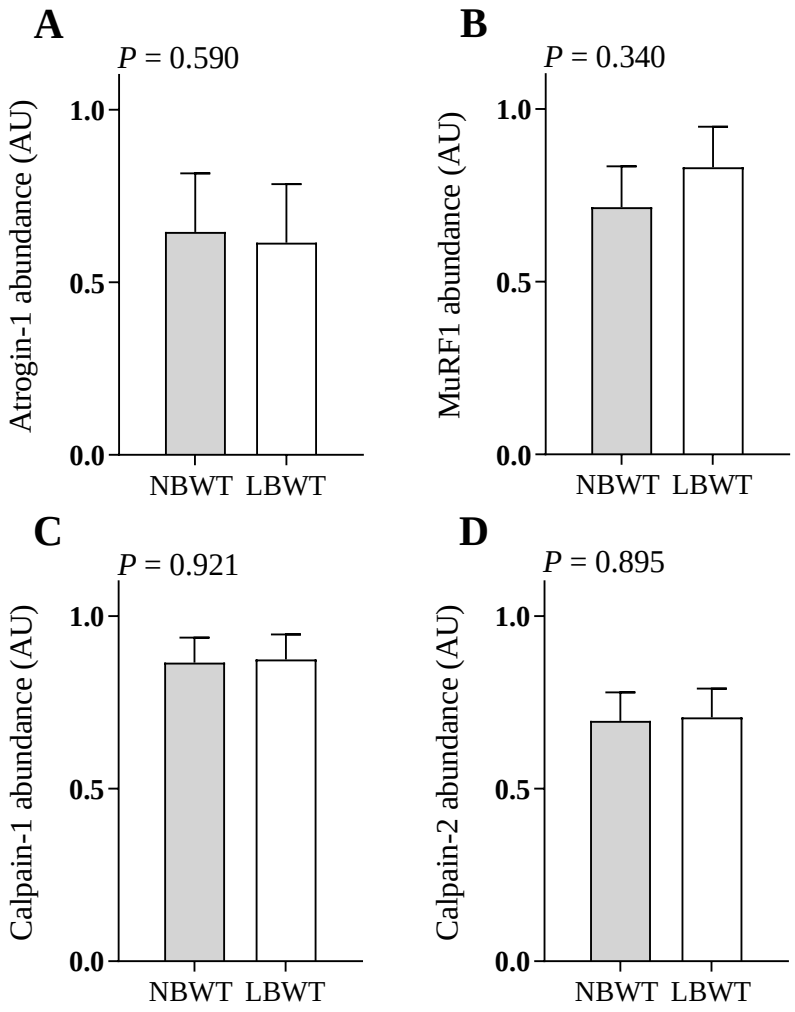

Figure 2-14. Total abundance of muscle-specific E3 ubiquitin ligases, Atrogin-1 (A) and MuRF1 (B), as well as calcium-dependent proteases, Calpain-1 (C), and Calpain-2 (D), in in the soleus of low birth weight (LBWT) and normal birth weight (NBWT) pigs. Results are means ± SE.

Chapter 3. Ractopamine Supplementation Induces Muscle Fiber Type Composition Shift but Does Not Promote Hypertrophy in Skeletal Muscle of Nursery-aged Pigs

ABSTRACT

Fiber type composition of a skeletal muscle is known to influence the rates of protein synthesis and degradation. We have previously reported downregulated protein synthesis signaling and a reduction in expression of type II MyHC isoforms in low birth weight (LBWT) neonatal pig compared with normal birth weight (NBWT) siblings. However, protein synthesis signaling and MyHC-II expression returned to normal in LBWT pigs at weaning (Seymour, Unpublished). The objectives of this study were to determine whether β-adrenergic agonist supplementation could enhance MyHC-IIB expression in nursery-age pigs, as well as to evaluate the influence of this fiber type transformation on the regulation of protein synthesis and degradation pathways. Twenty-four crossbred pigs (n = 12) were weaned and randomly assigned to one of two experimental diets: a corn and soybean-based nursery diet (CON) or nursery diet supplemented with 9 ppm ractopamine hydrochloride (RAC). Pigs were fed for 28 d before muscle and organs were collected, weighed, and stored until analyses. Although RAC supplementation increased expression of MyHC-IIB ($P < 0.01$) and improved translational capacity ($P < 0.05$), there was no effect of RAC on body or organ weights. In the longissimus dorsi (LD), RAC enhanced S6K1 phosphorylation ($P < 0.05$) and tended to reduce 4EBP1 abundance ($P = 0.065$). Although these changes should lead to greater protein translation, this effect was likely negated by a reduction in S6K1 abundance ($P \leq 0.05$) in RAC pigs. Additionally, the LD of RAC supplemented pigs had lower abundances of Calpain-1 ($P = 0.063$) and Calpain-2 ($P < 0.05$) with no change in the expression of Atrogin-1 and MuRF1. These data suggest a reduction in the degradation of myofibrillar proteins in skeletal muscles. However, there was no change in protein deposition as indicated by the lack of skeletal muscle hypertrophy. The results of this study indicated that a

fiber type transformation alone is not enough to alter protein turnover signaling in favor of hypertrophy.

INTRODUCTION

In mammals, four types of mature muscle fibers have been classified based on its predominantly expressed myosin heavy chain (MyHC) protein isoform (type I, IIA, IIX, or IIB) (Schiaffino and Reggiani, 2011). Each of these isoforms is associated with a variety of contractile, metabolic, and morphological characteristics (Lefaucheur et al., 2002). Briefly, type I fibers are "slow-twitch" fibers that are highly fatigue-resistant, have the greatest oxidative capacity, and are frequently found in postural muscles. Type II or "fast-twitch" fibers display a wider range of characteristics. Type IIA fibers are the most like type I fibers, have relatively high oxidative capacity and a moderate fatigue-resistance. At the other end of the spectrum, type IIB fibers are highly glycolytic, have the greatest power output, and are found in high concentrations in phasic muscles. Finally, type IIX fibers are intermediate between type IIA and IIB fibers for most characteristics.

Since functional demands on a muscle may change over time, an important quality of skeletal muscle is ability to shift fiber type composition in response to various hormonal or mechanical stimuli (Pette and Staron, 1997). This is especially true during the rapid skeletal muscle growth that occurs in the early postnatal period. For example, the MyHC IIB isoform is not expressed in porcine muscle before birth (Chang and Fernandes, 1997), thus throughout the early postnatal stage many muscles become increasingly glycolytic (Katsumata et al., 2017). This transformation is advantageous during rapid muscle growth. Although the rate protein synthesis is correlated with type I fiber content of a muscle (Garlick et al., 1989), oxidative muscles also display greater rates of protein degradation compared with glycolytic muscles (Bates and Millward, 1983; Kelly et al., 1984). Slower rates of protein turnover allow glycolytic fibers to be more efficient at depositing protein compared with oxidative fibers (van Wessel et al., 2010).

Low birth weight (LBWT) neonatal pigs are subject to slower rates of skeletal muscle growth due to downregulation of the protein synthesis signaling pathway (Fidancı et al., 2010; Chen et al., 2017). Additionally, expression of type II MyHC isoforms is less in LBWT compared with their NBWT siblings (El-Kadi et al., 2018). However, our data suggest that protein synthesis is upregulated in the glycolytic LD of 21-day old LBWT pigs following compensatory growth. In addition MyHC expression was similar in LBWT to that of their normal birth weight (NBWT) siblings (Seymour, Unpublished). It is not yet clear whether this reduction in protein synthesis in LBWT pig muscle occurred as a result of fiber type shift or was confounded by other intrinsic changes in the LBWT pig muscles.

Evidence suggest that fiber type transformation could be induced through dietary supplementation of β-adrenergic agonists (βAAs) like Ractopamine hydrochloride (RAC). Dietary RAC supplementation not only enhances muscle hypertrophy as a result of greater protein synthetic rates in finishing pigs (Bergen et al., 1989; Adeola et al., 1992), but also promotes greater expression of MyHC-IIB (Depreux et al., 2002). In this study, we hypothesized that RAC supplementation would shift skeletal muscle fiber type composition in young pigs to a more glycolytic phenotype and alter the signaling mechanisms regulating protein turnover in a manner that favors protein deposition. Our objectives were to: 1) determine whether β-adrenergic agonist supplementation would enhance MyHC-IIB expression in nursery-age pigs, and 2) evaluate the influence of this fiber type transformation on the regulation of protein synthesis and degradation pathways.

MATERIALS AND METHODS

Animals and Sample Collection

At 21-d of age, 24 crossbred male and female pigs of similar body weight were weaned from their sows and transported to an environmentally-controlled room. After a period of acclimation, pigs were weighed, individually-housed, and allotted to one of two experimental diets: a corn and soybean-based nursery diet (CON, 8.7 ± 0.181 kg) and the same nursery diet supplemented with 9 ppm ractopamine hydrochloride (RAC, 8.8 ± 0.176 kg). Pigs had *ad libitum* access to feed and water. Feed refusals were collected daily and body weights were recorded at the beginning of each week. Following 28-d of feeding the experimental diets, pigs were weighed and euthanized for tissue collection. Fractional growth was calculated from the ratio of body weight at time t to body weight at the initiation of feeding. Feed efficiency was calculated as the ratio of weekly gain in body weight to weekly feed intake. All procedures involving animals were approved by the Virginia Tech Institutional Animal Care and Use Committee (IACUC).

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from longissimus dorsi (LD) tissue using the Direct-zol RNA Miniprep Kit (ZYMO Research, Orange, CA), followed by reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time quantitative PCR (qPCR) reactions were performed with gene-specific primers (Table 3-2) and SYBR chemistry on an ABI 7500 Fast Real-time PCR cycler (Applied Biosystems). Relative mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method after normalization with the geometric mean of TOP2B and PPIA expression. Housekeeping genes were selected from candidate genes

using the NormFinder algorithm (Version 0.953) (Andersen et al., 2004). All primer sets were evaluated for adequate efficiency and validated by sequencing before use in this study.

Total Protein Content and Translational Capacity

Total protein content was determined by perchloric acid (PCA) extraction. 100 mg of frozen LD muscle was homogenized in cold 0.2 M PCA, followed by two additional washes using the same solution. The pellet was resuspended in 0.3 M sodium hydroxide and incubated at 37°C for 1 hr. Total protein concentration was determined using Bicinchoninic Acid Protein Assay Kit (Thermo Fisher Scientific, Wilmington, DE). The ratio of RNA to total protein per mg of tissue was used to estimate translational capacity (Fiorotto et al., 2014).

Protein Abundance and Phosphorylation

Western blotting was used to examine protein abundance and phosphorylation status in LD muscle samples. Approximately 100 mg of tissue was homogenized in a buffer (1.7 w/v), containing 100 mM potassium chloride, 50 mM β-glycerophosphate, 50 mM sodium fluoride, 20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 2 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 0.2 mM ethylenediaminetetraacetic acid (EDTA), and protease inhibitor cocktail (1:150; P8340, Sigma, St. Louis, MO). Tissue lysates were normalized for protein content before diluting (1:1) in 2× Laemmli Sample Buffer (Bio-Rad, Hercules, CA) and boiled for 5 min.

Equal volumes of samples were separated by polyacrylamide gels electrophoresis and transferred to a polyvinylidene difluoride membrane (Thermo Scientific). Membranes were

blocked with 3% bovine serum albumin (Research Products International Corp, Mount Prospect, IL) in tris-buffered saline with 0.1% Tween-20 (TBST) for 2 h. Membranes were incubated with protein-specific primary antibodies (Table 3-3) overnight at 4°C with gentle rocking. After extensive washing in TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse or goat anti-rabbit IgG-HRP conjugate, Bio-Rad) for 1 h at room temperature. Membranes were washed again in TBST and developed with Amersham™ ECL Western blotting detection reagents (GE Healthcare, Piscataway, NJ), and imaged for chemiluminescence using a BioDoc digital imager (Bio-Rad). Band optical density was obtained using Image Lab 4.0 software (Bio-Rad). Total intensity values were normalized against abundance of α-tubulin.

Statistical Analysis

Data were analyzed using PROC MIXED in SAS version 9.3 (SAS Institute Inc., Cary, NC). Diet was used as the main effect while sow number and sex were treated as random effects. Data are presented as the least square means ± standard error. Differences are considered significant at $P \leq 0.05$, unless otherwise noted.

RESULTS

Body and Muscle Weights

The absolute body weights (Fig. 3-1A) of pigs fed the control diet and diet containing RAC significantly increased over the experimental period ($P < 0.0001$), however there was no difference in body weight over time between the dietary groups. While fractional growth (Fig. 3-1B) increased over the experimental period ($P < 0.0001$), there was no significant difference between the diets. Furthermore, there we no difference in the weights of dissected muscles (Fig. 3-2A-D).

Voluntary Feed Intake

The amount of feed consumed daily (Fig 3-3A) by pigs increased over the experimental period ($P < 0.0001$). However, there was no difference in voluntary feed intake between diets. Additionally, feed efficiency (Fig 3-3B) increased between weeks 1 and 2 and then was no different for the remainder of the experimental period ($P < 0.0001$). There was no effect of diet on feed efficiency.

RNA, Protein, and Translational capacity

RAC supplementation increased RNA content (Fig. 3-4A) per mg of tissue while total protein content (Fig. 3-4B) was unaffected. Translational capacity (Fig. 3-4C), calculated by the ratio of total RNA to total protein, was significantly higher in the LD of pigs fed RAC compared with pigs fed the CON diet.

mRNA Expression

There were no differences in the expression of MyHC I or MyHC IIA mRNA (Fig. 3-5A and B) between pigs fed the control or RAC-containing diets. However, expression of MyHC IIB (Fig. 3-5D) was greater ($P < 0.01$) in pigs fed the RAC diet while expression of MyHC IIX (Fig. 3-5C) was numerically lower ($P = 0.057$). Additionally, mRNA expression of the β_2 adrenoceptor (Fig. 3-6) was not affected by diet.

Translation Initiation Signaling

Total abundance of Akt (Fig. 3-7A) was reduced in muscles of pigs fed the RAC-supplemented diet. In contrast, phosphorylation of Akt at Ser473 or Thr308 (Fig. 3-7B and C) was not different between diets. Total expression of S6K1 (Fig. 3-8A) was greater ($P = 0.05$) in

CON pigs while phosphorylation (Fig. 3-8B) was greater ($P < 0.05$) in RAC-supplemented pigs. Though the abundance of 4E-BP1 tended to be lower in RAC compared with CON pigs (Fig. 3-8C), there was no difference in 4E-BP1 phosphorylation between diets.

Protein Degradation Pathways

Abundance of calcium-dependent proteases Calpain-1 and -2 was measured to evaluate protein degradation signaling. Expression of Calpain-1 (Fig. 3-9A) tended ($P = 0.063$) to be less for RAC fed pigs compared with those in the CON group, while Calpain-2 expression (Fig. 3-9B) was reduced ($P < 0.05$) in pigs fed the RAC diet. However, protein expression of skeletal muscle-specific E3 ubiquitin ligases Atrogin-1 and MuRF1 were not affected by diet (Fig. 3-10A and B).

DISCUSSION

The current paradigm is that fiber type composition in skeletal muscle has a substantial effect on the ability of a muscle to efficiently deposit proteins (van Wessel et al., 2010). For example, oxidative muscles display greater rates of protein synthesis and degradation than glycolytic muscles (Bates and Millward, 1983; Kelly et al., 1984), which results in faster turnover of the protein pool but limits net accretion. Moreover, whole muscle growth rates are greater in muscles with high concentrations of glycolytic fibers (Ono et al., 1993; Rehfeldt et al., 2008; Wimmers et al., 2008). In this regard, we have previously reported reduced expression of type II MyHC isoforms in skeletal muscles of LBWT neonatal pigs compared with their NBWT siblings (El-Kadi et al., 2018). This reduction in type II MyHCs, especially for the glycolytic isoforms, may contribute to the slow and inefficient growth of LBWT pigs. Furthermore, we have also reported that the accelerated growth of LBWT pigs during the suckling period coincided with MyHC expression being restored to a level comparable with their NBWT siblings (Seymour el al., Unpublished). However, it is unclear whether changes in muscle fiber type independently from

other physiological differences could explain this change in growth rates. In this study, we set out to determine whether RAC-induced slow-to-fast fiber type transformation would affect protein turnover signaling in the skeletal muscle of young pigs.

βAAs are well known for their ability to improve growth performance and body composition in livestock species (Bergen and Merkel, 1991). For example, in finishing lambs and pigs, βAA administration improves weight gain compared with control animals (Kim et al., 1987; Carr et al., 2005). In contrast, our data indicated that while growth was observed throughout the 28-d feeding period, there was no effect of RAC on weight gain or fractional body weight. Although feeding RAC for 28 d had no effect on body weight, differences in body composition could obscure changes in lean tissue accretion. This is why skeletal muscles were dissected and weighed as a surrogate measure for lean tissue deposition. There were no differences in longissimus dorsi (LD), soleus, semitendinosus, or masseter muscles weights, indicating that feeding weaned pigs RAC for 28 d did not promote lean tissue growth. This is in contrast to previous reports, where βAAs improved lean tissue mass in pigs (Carr et al., 2005), lambs (Lopez-Carlos et al., 2011), and even humans with muscular dystrophy (Skura et al., 2008). Furthermore, despite evidence of improved feed efficiency during the finishing phase in lambs (Baker et al., 1984), cattle (Vestergaard et al., 1993), and pigs (Jones et al., 1985), there were no differences in feed intake or feed efficiency between diets throughout the experimental period. It has been reported that animal maturity can influence the efficacy of βAA treatment (Fiems, 1987). For example, a low dose of clenbuterol enhances weight gain in lambs weighing 40 kg whereas the same dose has no effect in lambs weighing 37.5 kg (Baker et al., 1984). Similarly, feeding of cimaterol improved carcass leanness in finishing-phase pigs (Jones et al., 1985) but had no effect on body composition when fed to pigs weighing only 10 kg (Mersmann et al., 1987). The lack of

an effect on muscle growth in the current study is likely to be related to age, however, we cannot exclude the dose or βAA potency as contributing factors.

Although there was no effect of RAC feeding on growth performance in nursery-age pigs, MyHC IIB expression in the LD was greater with a concomitant reduction of MyHC IIX expression in RAC-supplemented than in CON pigs. These changes in MyHC expression are consistent with reports following βAA administration in finishing pigs (Depreux et al., 2002), mice (Pellegrino et al., 2004), and cattle (Weber et al., 2012), where βAA supplementation initiated an increase in expression of the most glycolytic isoform and a reduction in one or all other isoforms, depending on the potency of the βAA used. With clear evidence that RAC feeding initiated the characteristic fiber type transformation in the muscle of young pigs, we sought to determine whether this change in MyHC expression corresponded with any changes in protein synthesis and degradation in muscles.

Postnatal hypertrophy occurs either through an increase in translational capacity or efficiency (Bush et al., 2003). βAAs increase muscle RNA content in rats (Eadara et al., 1989), lambs (Beermann et al., 1987), and pigs (Helferich et al., 1990). Our findings are in support of previous data, where pigs in the RAC-supplemented group had a 43% increase in RNA content over the CON diet in the LD. Since 80-85% of cellular RNA is believed to be ribosomal RNA (Henshaw et al., 1971), this would suggest that the RAC-supplemented pigs have greater translational capacity compared with pigs fed the CON diet. Although an increase in synthetic capacity should improve the rate of protein synthesis through greater ribosome abundance, the growth performance data suggest that protein deposition was not affected by RAC supplementation.

To understand the lack of skeletal muscle growth despite improvements to synthetic capacity, we profiled the expression and activation of components related to protein synthesis signaling. In skeletal muscles of finishing phase animals, the use of βAAs improves fractional protein synthesis rate by promoting translation initiation (Adeola et al., 1992; Nash et al., 1994). βAAs stimulate protein synthesis via promotion of AKT activity in the IGF-1-PI3K-mTORC1 pathway (Ito et al., 2019), which leads to greater formation of translationally-competent ribosomes. In the current study, however, AKT abundance was reduced following RAC-supplementation. While the reason behind this reduction has yet to be elucidated, a reduction in expression of AKT would likely reduce mTORC1 activation following a hypertrophic stimulus (Bodine et al., 2001; Roux and Topisirovic, 2012). Protein abundance of mTORC1 downstream effectors, ribosomal protein S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4EBP1) were not affected by dietary RAC supplementation. Interestingly, while 4EBP1 phosphorylation was also similar for RAC-supplemented and CON pigs, phosphorylation of S6K1 was greater following RAC supplementation.

There are two plausible explanations for this enhanced S6K1 phosphorylation. One is that S6K1 phosphorylation could simply be due to the βAA supplementation (Kline et al., 2007). βAAs cross-talk with the IGF-1-PI3K-mTORC1 pathway via two mechanisms. The first is through the activation of phosphoinositide 3-kinase (PI3K) by the adrenergic receptor's Gβγ subunit, where PI3K phosphorylates and activates AKT at Thr^{308} through the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3) (Kline et al., 2007). The other mechanism by which the adrenergic and translation signaling pathways interact is through the production of cyclic AMP (cAMP) (Brennesvik et al., 2005). Binding of a βAA to the adrenoceptor activates the G-protein subunit (Gα), which stimulates adenylate cyclase (AC) to

convert ATP to cAMP (Sato et al., 2011). Accumulation of cAMP potentiates the insulin-stimulated activation of AKT at Ser^{473} via a cAMP-binding protein (Epac) (Brennesvik et al., 2005). However, phosphorylation of AKT at Ser^{473} or Thr^{308} were similar between diets, suggesting no difference in stimulation of either mechanism as the result of RAC supplementation. In fact, the previously mentioned reduction in AKT abundance seen in RAC pigs would suggest stimulation of the translation signaling pathway is downregulated, further reducing the likelihood that the enhanced phosphorylation of S6K1 was the result of βAA administration. The second explanation for this difference in S6K1 phosphorylation is that it may be correlated with the slow-to-fast fiber type shift. While it has yet to be determined whether oxidative and glycolytic fibers express phosphorylated S6K1 similarly, it has been suggested that glycolytic fibers have a greater potential to phosphorylate S6K1 following hypertrophic stimuli compared with oxidative fibers (van Wessel et al., 2010).

In addition to evaluating the regulation of protein synthesis, we also profiled two key protein degradation pathways known to be affected by βAA administration, the calpain/calpastatin and the ubiquitin proteasome systems. In both rabbits and finishing lambs, βAA supplementation reduces expression of one or both calcium-dependent proteases (Forsberg et al., 1989; Pringle et al., 1993). In agreement with these studies, our data suggest that RAC-fed pigs reduced the abundance of Calpain-1 and -2 compared with pigs fed the CON diet. While the mechanism has yet to be fully elucidated, βAAs are thought to inhibit the calpain system in skeletal muscle through cAMP production (Bardsley et al., 1992). To this end, *in vitro* data suggest that that phosphorylation of protein kinase A (PKA) following accumulation of cAMP inhibits epidermal growth factor (EGF)-induced calpain activation in fibroblasts (Shiraha et al., 2002). However, whether a similar mechanism occurs in the skeletal muscle during RAC supplementation is still

unclear. These calcium-dependent cysteine proteases facilitate the degradation of myofibrillar proteins by breaking down the sarcomeric scaffold (Smith and Dodd, 2007; Sorimachi and Ono, 2012). While lower Calpain abundance should, in theory, slow the degradation of myofibrillar proteins by leaving the sarcomeric structure intact, the lack of skeletal muscle growth suggests that these changes could not shift the balance of protein turnover in favor of protein accretion.

Although others have reported *in vitro* and *in vivo* that βAA use suppresses expression of the ubiquitin ligases Atrogin-1 and MuRF1 (Kline et al., 2007; Wannenes et al., 2012; Shimamoto et al., 2016), in the current study expression of Atrogin-1 and MuRF1 was not affected by RAC supplementation. One possible explanation for this discrepancy is that these studies clenbuterol was used rather than RAC. Clenbuterol is known to be more potent than RAC due to a longer half-life and greater oral bioavailability (Smith, 1998). Alternatively, this lack of response could be a consequence of the duration RAC was provided. The efficacy of βAAs is known to diminish with increasing exposure due to the auto-regulatory process of receptor desensitization (Johnson, 1998; Almeida et al., 2012), a process which includes inactivation through phosphorylation, receptor sequestration, and downregulation of adrenoceptor mRNAs (Hausdorff et al., 1990). Although in the current study there were no differences in mRNA expression of the β_2-adrenoceptor between diets, we cannot rule out the possibility that the adrenergic receptors were either inactive or not available for ligand binding as these were not measured.

In conclusion, our results show that RAC induced a fiber type transformation in the skeletal muscle of nursery-age pigs, as well as increased muscle translational capacity. Though these changes were accompanied by some regulatory signaling modulation in both protein synthesis and degradation-related pathways, there was no evidence of noticeable skeletal muscle hypertrophy.

These findings suggest that a slow-to-fast fiber type shift without the influence of other physiological factors does not improve skeletal muscle hypertrophy in young pigs.

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Table 3-1. Nutrient composition of experimental diets

	Diet	
	Control	Supplemented
Ractopamine hydrochloride, *ppm*	-	9.0
Metabolizable energy (ME), *kcal/kg*	3321.7	3301.1
Moisture, *% DM*	9.1	10.1
Crude protein, *% DM*	24.1	24.8
Crude fat, *% DM*	4.2	4.6
Non-fiber carbohydrates, *% DM*	56.0	55.1
Ash, *% DM*	5.5	5.6
Calcium, *% DM*	0.70	0.68
Phosphorus, *% DM*	0.66	0.69
Potassium, *% DM*	0.90	0.94
Magnesium, *% DM*	0.20	0.21
Sodium, *% DM*	0.38	0.35

Table 3-2. Nucleotide sequences for real-time PCR primers

Target	Direction	Primer Sequence	Accession No.
B_2 adrenergic receptor	Forward	5'- GTC GTG TCG GGC CTT ATC TC -3'	NM_001128436.1
	Reverse	5'- ACA CGA TGG AAG AGG CGA TG -3'	
Cyclophilin A	Forward	5'- CGT CTT CTT CGA CAT CGC CG -3'	NM_214353.1
	Reverse	5'- GAA GTC ACC ACC CTG GCA CAT AAA T -3'	
Myosin heavy chain I	Forward	5'- CTG TCC AAG TTC CGC AAG GT -3'	NM_213855.2
	Reverse	5'- TCC TCG TTC AAG CCC TTG GT -3'	
Myosin heavy chain IIA	Forward	5'- CTC ACT TGC TAA GAA GGA CCT CTG -3'	NM_214136.1
	Reverse	5'- GCC TCA ATG CGC TCC TTT TC -3'	
Myosin heavy chain IIB	Forward	5'- GTA CAT CTA GTG CCC TGC TGC -3'	NM_001123141.1
	Reverse	5'- CCC TGC TTC CGT CTT CAC TG -3'	
Myosin heavy chain IIX	Forward	5'- ACA GGA ACA CCC AAG GCA TC -3'	NM_001104951.2
	Reverse	5'- GAG CTC CTG TTC TGC GAC TT -3'	
DNA topoisomerase 2β	Forward	5'- AAC TGG ATG ATG CTA ATG ATG CT -3'	NM_001258386.1
	Reverse	5'- TGG AAA AAC TCC GTA TCT GTC TC -3'	

Table 3-3. Primary antibodies used for western blotting

Antibody	Company
4EBP1	#A300-501A; Bethyl Laboratories, Montgomery, TX
Atrogin-1	#AP2041; ECM Biosciences, Versailles, KY
Calpain 1	#2556; Cell Signaling Technology, Danvers, MA
Calpain 2	#373967; Santa Cruz Biotechnology, Dallas, TX
p70 S6 kinase	#2708; Cell Signaling Technology, Danvers, MA
phospho-Akt (Ser^{473})	#9271; Cell Signaling Technology, Danvers, MA
phospho-Akt (Thr^{308})	#AP0304; ABclonal, Woburn, MA
phospho-4EBP1 (Thr^{46})	#44-1170G; Invitrogen, Camarillo, CA
phospho-S6K1 (Thr^{389})	#07-018-I; Millipore, Temecula, CA
PKB/Akt	#9272; Cell Signaling Technology, Danvers, MA
α-Tubulin	#H300; Santa Cruz Biotechnology, Dallas, TX

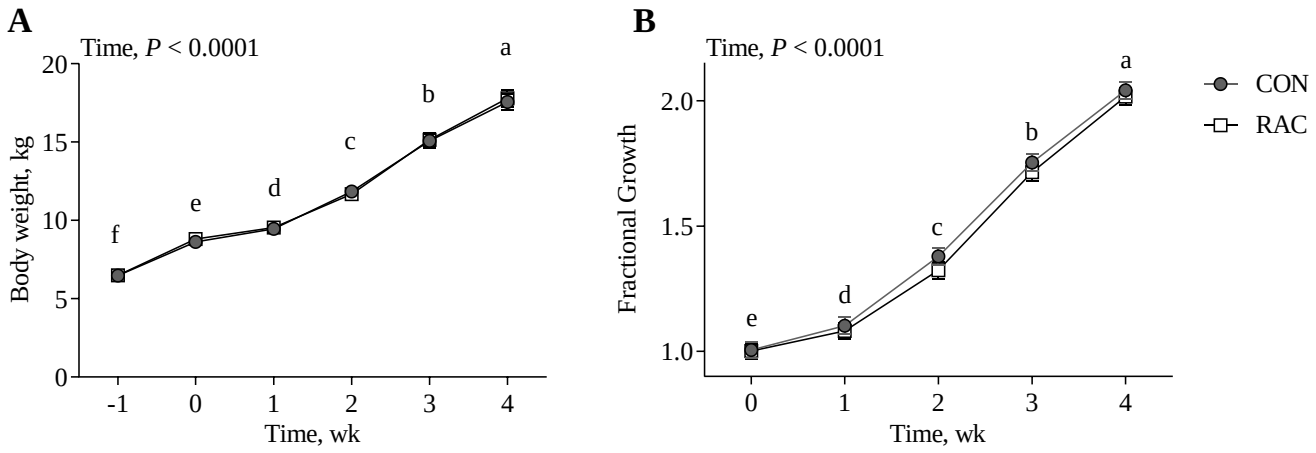

Figure 3-1. Absolute body weight (**A**) and fractional growth (**B**) over the experimental period for pigs fed the control or ractopamine-supplemented diet. Results are means ± SE. Differing letters indicate significant difference ($P \leq 0.05$).

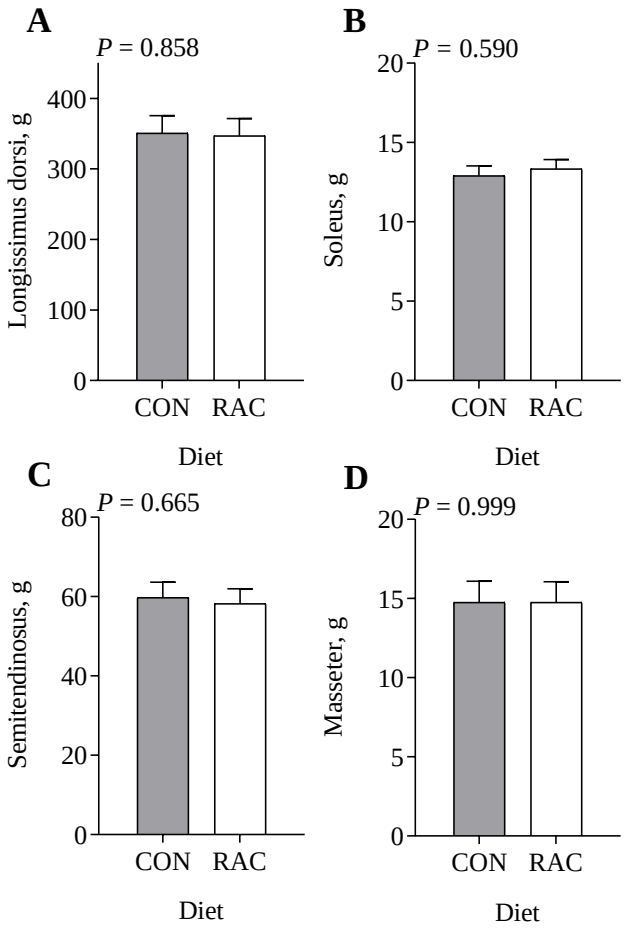

Figure 3-2. Longissimus dorsi (**A**), soleus (**B**), semitendinosus (**C**), and masseter (**D**) muscle weights from pigs fed the control or ractopamine-supplemented diet. Results are means ± SE.

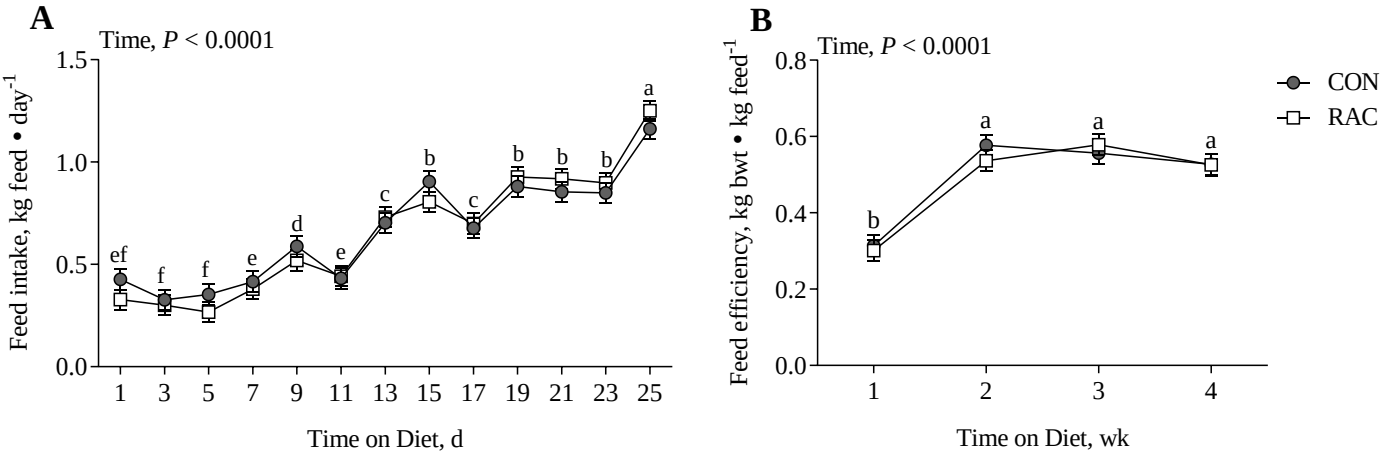

Figure 3-3. Feed intake per day (**A**) and feed efficiency by week (**B**) over the experimental period for pigs on the control or ractopamine-supplemented diet. Results are means ± SE. Differing letters indicate significant difference ($P \leq 0.05$).

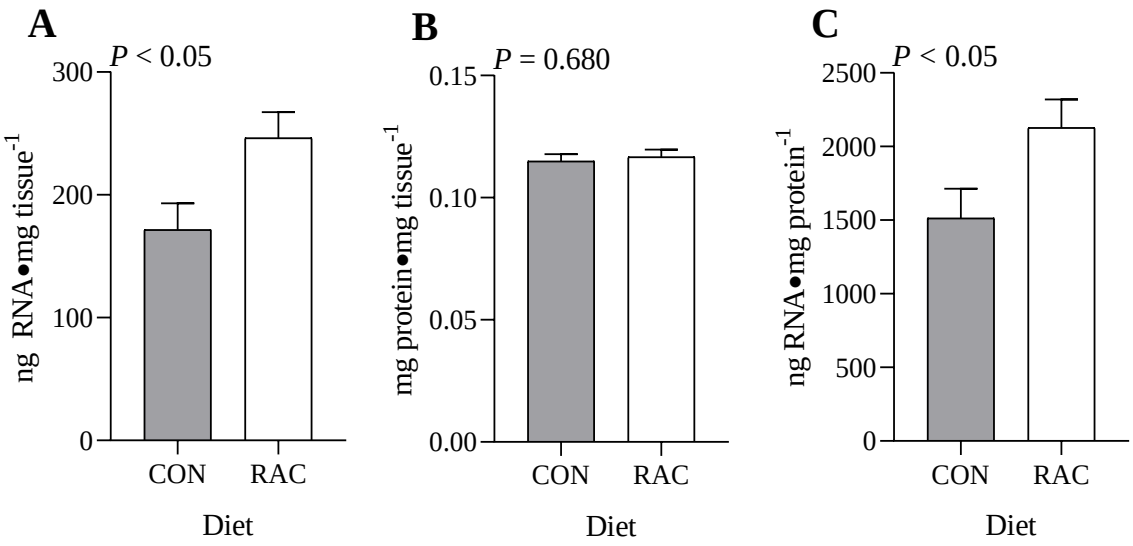

Figure 3-4. RNA content (**A**), protein content (**B**), and translational capacity (**C**) of the longissimus dorsi of pigs fed the control or ractopamine-supplemented diet. Results are means ± SE.

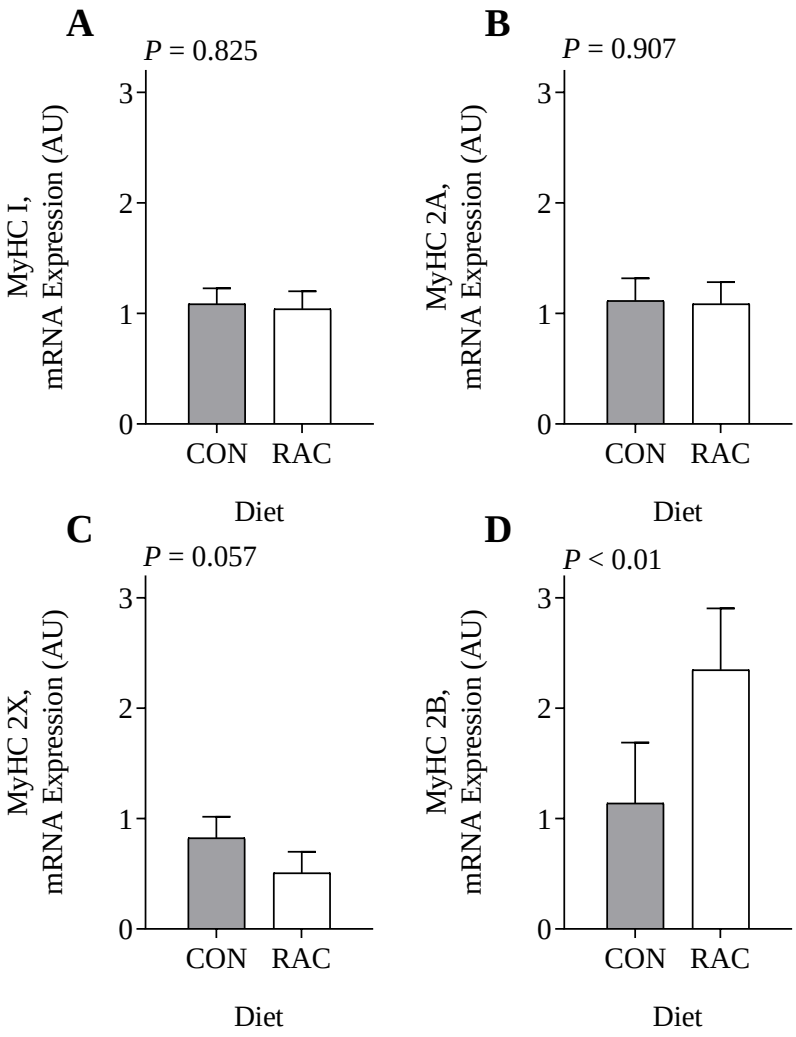

Figure 3-5. mRNA expression of the myosin heavy chain (MyHC) isoforms in the longissimus dorsi muscle of pigs fed the control or ractopamine-supplemented diet. Results are means ± SE.

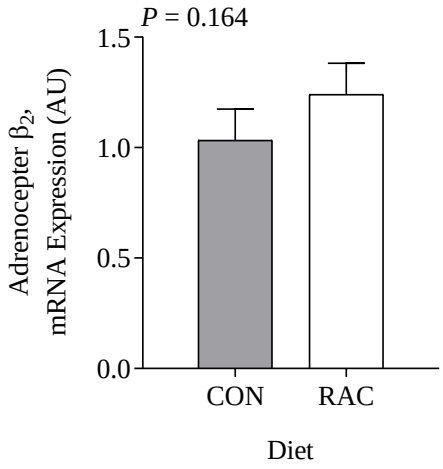

Figure 3-6. mRNA expression of the β_2-adrenoceptor in the longissimus dorsi muscle of pigs fed the control or ractopamine-supplemented diet. Results are means ± SE.

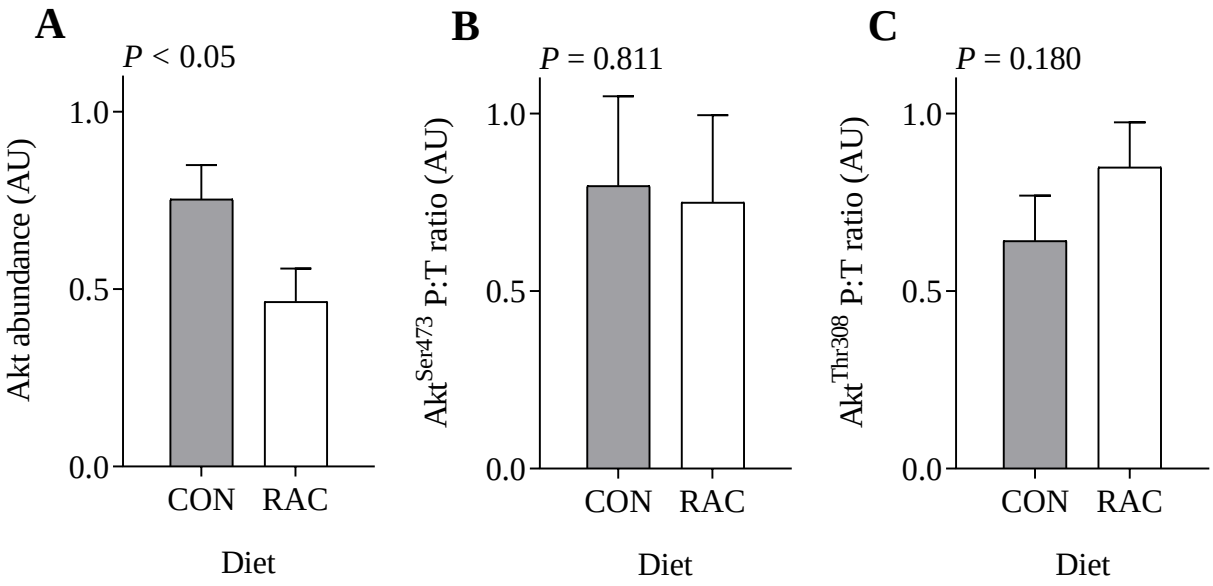

Figure 3-7. Total abundance of Protein Kinase B (Akt) (**A**), phosphorylation of Akt^{Ser473} (**B**), and phosphorylation of Akt^{Thr308} (**C**) in the longissimus dorsi of pigs fed the control or ractopamine-supplemented diet. Results are means ± SE. Asterisks indicate significant difference ($P \leq 0.05$).

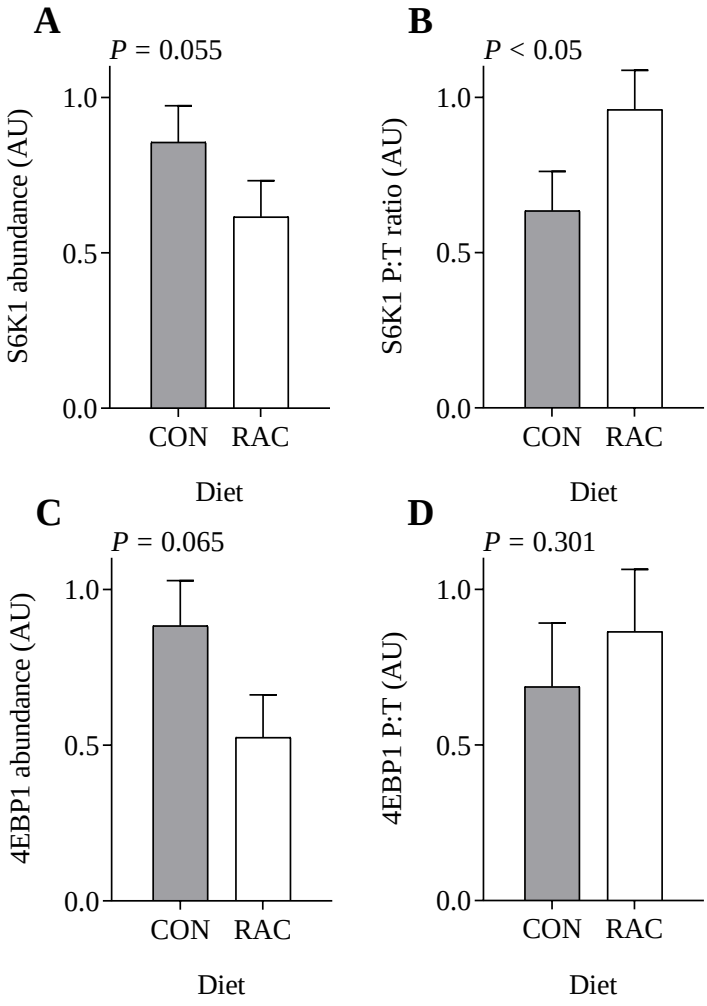

Figure 3-8. Total protein abundance of Eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) (**A**), total abundance of ribosomal protein S6 kinase 1 (S6K1) (**B**), phoshorylation of 4EBP1 (**C**), and phosphorylation of S6K1 (**D**) in the longissimus dorsi muscle of pigs fed the control or ractopamine-supplemented diet. Results are means ± SE.

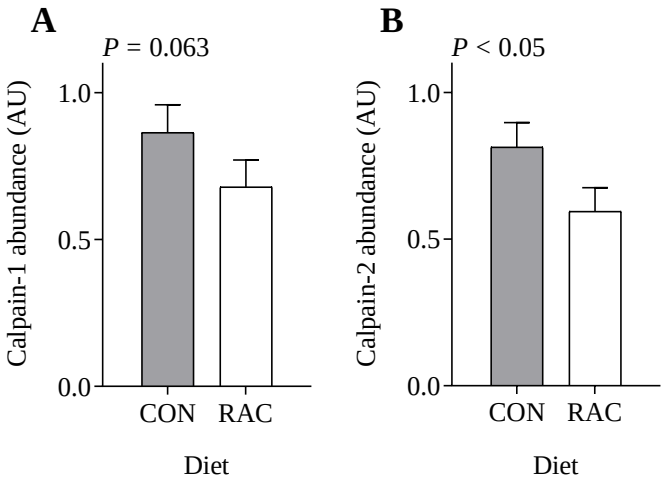

Figure 3-9. Total abundance of Calpain-1 (A) and Calpain-2 (B) in the longissimus dorsi of pigs fed the control or ractopamine-supplemented diet. Results are means ± SE.

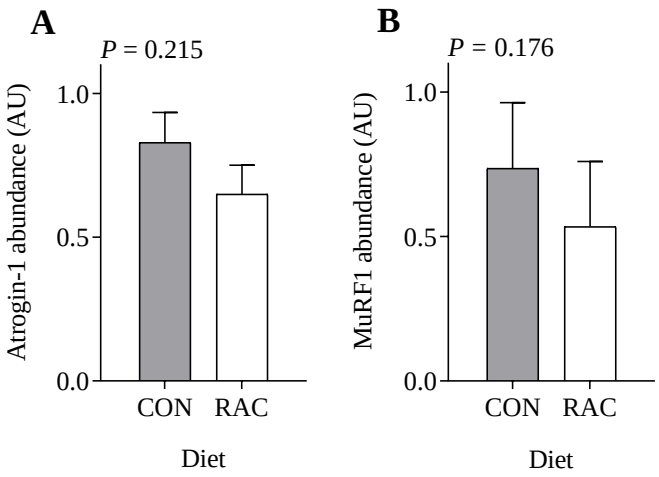

Figure 3-10. Total abundance of muscle-specific E3 ubiquitin ligases, Atrogin-1 (A) and MuRF1 (B), in the longissimus dorsi of pigs fed the control or ractopamine-supplemented diet. Results are means ± SE.

Chapter 4. Summary

The fiber type composition of skeletal muscle not only dictates important qualities like force production, fatigue resistance, and metabolic preferences, but it may also influence a muscle's ability to grow. Given that myocyte hyperplasia in mammalian skeletal muscles ceases around the time of birth, any changes to muscle mass that occur postnatally are the result of muscle fiber hypertrophy, a process driven by myonuclear incorporation and a positive balance between the rates of protein synthesis and degradation. Thus, the hypothesis of this thesis was that fiber type composition will differentially influence the regulation of signaling pathways related to protein synthesis and degradation, and that such changes will lead to differences in skeletal muscle hypertrophy in growing pigs. With this research, we aimed to identify differences in protein synthesis and degradation mechanisms across various skeletal muscles during a period of accelerated compensatory growth, as well as to evaluate the influence of a fiber type transformation on the regulation of protein turnover.

The regulation of protein synthesis and degradation pathways was investigated in two skeletal muscles of low birth weight (LBWT) and normal birth weight (NBWT) pigs at weaning. The two muscles used were the longissimus dorsi (LD), a fast-twitch glycolytic muscle, and the soleus (SOL), a slow-twitch oxidative muscle. Body weight LBWT pigs as a proportion of the weight of their NBWT siblings increased from 50% at birth to approximately 65% at 21 d of age, indicating an accelerated growth rate in LBWT pigs compared with NBWT siblings during the suckling period. Rapid growth of the LD contributed to this accelerated weight gain and was likely due to an upregulation of the protein synthesis signaling pathway, more specifically related to mRNA translation initiation. While we had hypothesized that this pathway would continue to be downregulated due to the persistent disparity in muscle mass between LBWT and NBWT

individuals, our results indicate that an upregulation of translation initiation signaling is likely behind the compensatory growth of non-oxidative muscles.

In the second study, regulation of the same protein synthesis and degradation pathways was investigated following a fiber type transformation induced by the beta-adrenergic agonist, ractopamine hydrochloride (RAC). We hypothesized RAC supplementation would shift skeletal muscle fiber type composition in young pigs to a more glycolytic phenotype and alter the signaling mechanisms regulating protein turnover in a manner that favors protein deposition. Although RAC supplementation did promote the characteristic oxidative-to-glycolytic shift commonly seen in older animals, there was no observable effect on muscle hypertrophy. This was despite the fact that RAC supplementation increased RNA content and improved translational capacity. In the translation initiation pathway, increased phosphorylation of S6K1 and lower abundance of 4EBP1 should, in theory, have promoted greater initiation of protein translation. However, this effect was likely negated by a reduced abundance of S6K1. As for protein degradation components, RAC reduced expression of the calcium-dependent proteases. While this could potentially slow the degradation of myofibrillar proteins, these changes were not enough to allow for significant muscle hypertrophy. The results of this study indicate that a fiber type transformation alone is not enough to alter protein turnover signaling so that muscle hypertrophy occurs.

The results of these studies underline the complexity of protein turnover regulation and the factors that affect it. Furthermore, our findings suggest the extent of muscle hypertrophy in growing pigs is not strictly dependent on fiber type composition. Considering oxidative fibers have greater translational capacity but lack the ability to make significant improvements to fiber diameter, a clear profile of the fiber-type specific differences in abundance of translation initiation and protein degradation signaling components would provide a better understanding of the

relationship between fiber type and hypertrophy. Additionally, if fiber type is not the sole determinant of which LBWT skeletal muscles contribute to compensatory growth, other nutritional factors should be considered, such as whether the glycolytic muscles of LBWT pigs on limit feeding still experience accelerated growth.