

Investigating metabolic regulation of livestock skeletal muscle

by

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Department of Animal Sciences and Industry
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Manhattan, Kansas

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Abstract

Global population growth and shifts in dietary preferences, particularly in developing regions, have placed unprecedented pressure on the livestock industry to increase lean muscle production. Concurrently, reductions in available arable land require animal production systems to generate greater outputs while utilizing fewer resources. As a result, improving livestock efficiency remains a critical priority for sustaining future agricultural productivity.

Skeletal muscle plays a central role in feed efficiency and overall productivity, accounting for approximately 45% of an animal's body mass and consuming the majority of dietary glucose. This highly dynamic tissue is composed of muscle fibers with distinct metabolic profiles that influence nutrient utilization, energy production, and growth potential. Therefore, the objective of this dissertation was to determine how genetic variation alters skeletal muscle metabolism and regulates substrate partitioning between glycolytic and oxidative pathways. To address this objective, two unique animal models were utilized across bovine and porcine species. Skeletal muscle was collected from muscles representing a range of metabolic phenotypes, including the *longissimus dorsi* (LD; glycolytic), *latissimus dorsi* (LAT; mixed), *semitendinosus* (ST; mixed), and *masseter* (MS; oxidative).

The first study investigated cattle harboring a naturally occurring mutation in the glycogen myophosphorylase *PYGM* gene, commonly referred to as "PYGM cattle," to determine how impaired glycogenolysis influences downstream metabolism. In this model, restricted glycogen breakdown altered pyruvate metabolism through shifts in lactate dehydrogenase (LDH) isoform abundance. Specifically, LDH α abundance increased in whole muscle preparations from the LD and MS of PYGM cattle compared to control cattle ($p < 0.05$); while mitochondrial fraction preparations increased the abundance of LDH β in PYGM cattle regardless of muscle ($p < 0.05$).

Although pyruvate entry into the tricarboxylic acid (TCA) cycle via pyruvate dehydrogenase (PDH) did not differ between genotypes ($p > 0.05$), pyruvate carboxylase (PC) abundance increased in the LD and LAT muscles of PYGM cattle ($p < 0.05$). Collectively, these findings indicate a shift in pyruvate partitioning toward anaplerotic pathways to support mitochondrial metabolism under conditions of limited glycogen availability.

The second study examined a pig model that was specifically bred to be heterozygous for two naturally occurring mutations in key metabolic genes. One mutation in the ryanodine receptor gene (*RYR1*), which is associated with the halothane-sensitivity locus “(*HAL*).” A second mutation in the *PRKAG3* gene, which regulates AMP-activated protein kinase gamma subunit (AMPK γ) and is referred to as an “RN” mutation. The HAL mutation alters calcium handling in skeletal muscle, promoting a more glycolytic phenotype; however, this mutation is also associated with adverse effects on animal survivability and meat quality at slaughter. The RN mutation alters cellular energy sensing through regulation of AMP and ADP levels, resulting in constitutive activation of AMPK and promoting a more oxidative skeletal muscle phenotype. In contrast to the *PYGM* cattle model, LDH α abundance did not differ among genotypes ($p > 0.05$), whereas LDH β abundance decreased in the MS muscle of WT, RN, and RN-HAL pigs compared to all other muscles within each genotype ($p < 0.05$). Additionally, no differences were observed in PDH or PC abundance among genotypes ($p > 0.05$). These data suggest that nutrient partitioning and handling still differs even when substrates are not limited.

Collectively, these studies demonstrate that genetic variation alters skeletal muscle metabolism by regulating pyruvate fate, thereby influencing substrate partitioning between glycolytic and oxidative pathways. Overall, this work provides new insight into the metabolic mechanisms underlying nutrient utilization and mitochondrial function, with implications for

improving feed efficiency, enhancing muscle growth, and advancing sustainability in livestock production systems.

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List of Abbreviations

ADP	Adenosine diphosphate
AKG	Alpha-ketoglutarate
AMPK	AMP-activated protein kinase
ATP	Adenosine Triphosphate
ATP5 β	ATP synthase subunit beta
AU	Arbitrary units
BF	Backfat thickness
Ca ²⁺	Calcium
CO ₂	Carbon dioxide
CS	Citrate synthase
CSA	Cross-sectional area
ETC	Electron transport chain
FAD/FADH ₂	Flavin adenine dinucleotide
F/G	Feed to Gain
GC-MS	Gas chromatography mass spectrometry
G6P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
HAL	Halothane; Heterozygous RyR1
LAT	Latissimus dorsi
LD	Longissimus dorsi
LDH α	Lactate dehydrogenase alpha
LDH β	Lactate dehydrogenase beta
LEA	Loin-eye area
MTBSTFA	N-tert-butyltrimethylsilyl-N-methyltrifluoroacetamide
NAD ⁺ /NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
OAA	Oxaloacetate
OCR	Oxygen consumption rate
PC	Pyruvate carboxylase

PDKs	Pyruvate dehydrogenase kinases
PFK	Phosphofructokinase
P/M	Pyruvate/Malate
PYGM	Myophosphorylase gene
RN	Rendement Napole; Heterozygous AMPK
RyR1	Ryanodine Receptor 1
SDH α	Succinate dehydrogenase alpha
S/R	Succinate/Rotenone
ST	Semitendinosus
STR	Semitendinosus- red portion
STW	Semitendinosus- white portion
tBDMS	Tertiary-butyldimethylsilyl
TCA	Tricarboxylic acid
UDP	Uridine diphosphate
WT	Wild-type

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believing in me even when I struggled to believe in myself. I am endlessly grateful for your strength, encouragement, and the life we are building together. I could not have done this without you, and I am so lucky to have you by my side. I love you.

Dedication

This dissertation is dedicated to my mom and dad, whose unwavering love, support and belief in me have made all of this possible. I love you both so much.

Chapter 1 - Literature Review

Introduction

Global demand for livestock-derived protein has placed unprecedented pressure on animal production systems to generate greater outputs while relying on fewer inputs. As the global population continues to increase, reductions in available arable land further intensify the challenge of sustaining efficient livestock production. Concurrently, patterns of meat consumption are shifting worldwide, with increasing affordability of animal protein in developing regions. Projections indicate that U.S. meat exports to emerging markets are expected to rise by nearly 24% by 2034 (Sands et al., 2023). In fact, pork exports are projected to rise substantially, increasing from 6.2 to 8.1 billion pounds by 2034 (Dolhman et al., 2025). To meet this growing demand, the beef industry, which currently produces approximately 86 million head of cattle, is anticipated to increase to 91.6 million head by 2034 (Dolhman et al., 2025). Similarly, the swine industry produced 28.5 billion pounds of pork in 2025, with production expected to increase by 10% over the next decade (Dolhman et al., 2025). To support these escalating production demands, the livestock industry has implemented numerous strategies designed to enhance growth performance, including genetic selection, nutrition, growth promotants, and feed additives (Wu et al., 2011; Khanal et al., 2019; Pfau et al., 2024; Van Eenennaam, 2025). While these approaches have been highly successful and remain essential for modern production systems, improvements in efficiency have begun to slow. For example, feed conversion ratios decreased by 26% in feeder-to-finish operations from 1992 to 2004, yet only improved by 3% between 2004 and 2009 (McBride and Key, 2013). This plateau suggests that current strategies may be approaching biological or technological limits. Therefore, identifying novel mechanisms that govern growth and nutrient utilization is essential for sustaining future improvements in livestock performance.

Skeletal Muscle and Its Implications on Livestock Animal Performance

When evaluating the overall performance of a livestock animal, skeletal muscle serves as the primary site of lean tissue accretion and is a major determinant of production efficiency (Lee et al., 2010; Picard et al., 2010). Nutrient absorption and partitioning are fundamental drivers of key production characteristics, including average daily gain, feed intake, and feed efficiency (Lee et al., 2010; Picard et al., 2010; Khanal et al., 2019; Merz and Thurmond, 2020; Pfau et al., 2024). Because skeletal muscle plays a central role in growth and efficiency, understanding the metabolic processes that regulate muscle development and nutrient partitioning is critical for enabling continued advancements in livestock performance. Skeletal muscle accounts for approximately 35 to 60% of total body mass in livestock species and represents a major site of whole-body glucose utilization, consuming nearly 75% of dietary glucose uptake (Ferrannini et al., 1988; Listrat et al., 2016; Merz and Thurmond, 2020). These observations highlight that a deeper understanding of how skeletal muscle partitions and utilizes nutrients may reveal novel opportunities to enhance growth efficiency and lean tissue accretion.

Skeletal muscle is a highly heterogeneous tissue composed of fibers that differ in contractile speed, metabolic properties, and functional characteristics. These fibers are classified based on contractile speed and type of metabolism ranging from slow-contracting, oxidative type I fibers to fast-twitch, predominantly glycolytic type II fibers (Gerrard and Grant, 2003). Type I fibers are characterized by high mitochondrial density, oxidative metabolism, and smaller fiber diameter; whereas, type II fibers exhibit a greater reliance on glycolytic metabolism and are commonly associated with increased fiber size and hypertrophic potential (Gerrard and Grant, 2003; Lee et al., 2010; Listrat et al., 2016). Importantly, skeletal muscle fibers are not metabolically static but rather possess the capacity to shift their metabolic characteristics in

response to functional demands, nutrient availability, and environmental stimuli (Gerrard and Grant, 2003; Listrat et al., 2016; Wang et al., 2024).

Metabolic plasticity enables skeletal muscle to dynamically alter its reliance on carbohydrates and lipids depending on substrate availability and physiological state (Listrat et al., 2016; Merz and Thurmond, 2020; Cao et al., 2021). In livestock species, these metabolic characteristics have important implications for growth performance and efficiency. Glycolytic muscle phenotypes have been associated with improved production efficiency across species (Yen et al., 2024), suggesting that shifts in muscle metabolism may influence nutrient utilization and growth trajectories. Nutritional and pharmacological interventions further support this relationship. For example, β -adrenergic agonists have been shown to enhance growth performance (Storlie, 2012) and promote fiber shifts, including increased abundance of glycolytic enzymes in pigs supplemented with ractopamine (Costa-Lima et al., 2015). Similarly, glycolytic enzyme expression has been reported to be elevated in high feed-efficient pigs compared with their low-efficiency counterparts (Fu et al., 2017). Collectively, these findings highlight the central role of skeletal muscle metabolism in regulating nutrient utilization, growth, and production efficiency. Therefore, a deeper understanding of the metabolic processes governing skeletal muscle function may reveal metabolic inefficiencies that can be targeted to further improve livestock growth and lean tissue accretion.

Glycogen and Glycolysis Pathways

Carbohydrates represent the primary and most abundant source of dietary energy in livestock production systems (Faulkner, 2004; Menegat et al., 2019). Consumption of high-energy diets promotes carbohydrate storage within skeletal muscle, primarily in the form of glycogen, which typically accounts for approximately 0.5–1.5% of muscle mass (Rosenvold et al., 2001).

Glycogen serves as a critical metabolic reserve, supporting both local energy demands and systemic glucose homeostasis (Patino and Orrick, 2025). Glucose derived from the diet is taken up by muscle and stored through the process of glycogenesis. Briefly, glucose is phosphorylated to glucose-6-phosphate, which is subsequently converted to glucose-1-phosphate via phosphoglucomutase (Chaudhry and Varacallo, 2026). Glucose-1-phosphate is then activated to uridine diphosphate (UDP)-glucose by UDP-glucose pyrophosphorylase, providing the substrate for glycogen synthase to elongate branched glycogen polymers (Nakrani et al., 2025).

The glycogen pool functions as a readily accessible energy source that can be rapidly mobilized to meet fluctuations in metabolic demand. This reserve is particularly important for sustaining muscle contraction, maintaining ion gradients, supporting calcium cycling, and ultimately preventing fatigue during periods of increased energetic stress (Jensen et al., 2011). When energy requirements increase, glycogen is degraded through glycogenolysis. This process is initiated by muscle glycogen phosphorylase (myophosphorylase), which catalyzes the release of glucose-1-phosphate from glycogen (Jensen et al., 2011; Patino and Orrick, 2025). Glucose-1-phosphate is then converted back to glucose-6-phosphate, allowing entry into glycolysis and downstream adenosine triphosphate (ATP)-generating pathways (Jensen et al., 2011; Patino and Orrick, 2025).

Regardless of whether glucose originates from dietary uptake or glycogenolysis, its subsequent metabolism is primarily governed by glycolysis, a central pathway responsible for rapid ATP generation and metabolic regulation in skeletal muscle (Chaudhry and Varacallo, 2025). Glycolysis involves the breakdown of a single glucose molecule, requiring an initial investment of two ATP molecules, while yielding two molecules of pyruvate, along with a net production of four ATP and reducing equivalents in the form of nicotinamide adenine dinucleotide (NADH)

(Chaudhry and Varacallo, 2025). This pathway provides a rapid mechanism for ATP generation, enabling skeletal muscle to respond quickly to fluctuations in energetic demand. Glycolysis is initiated by the phosphorylation of glucose to glucose-6-phosphate, a reaction catalyzed by hexokinase (Chaudhry and Varacallo, 2026). Glucose-6-phosphate occupies a critical metabolic branch point, as it may either proceed through glycolysis or be diverted into the pentose phosphate pathway via glucose-6-phosphate dehydrogenase (Wagner et al., 1978; Chaudhry and Varacallo, 2025). Entry into the pentose phosphate pathway supports the generation of nicotinamide adenine dinucleotide phosphate (NADPH), which is essential for reductive biosynthesis and redox balance, as well as ribose-5-phosphate, a precursor for nucleotide synthesis (Wagner et al., 1978). Notably, activation of this pathway has been associated with periods of muscle remodeling, including regeneration and hypertrophy (Wagner et al., 1978; Valentino et al., 2021). When glucose-6-phosphate continues through glycolysis, it is converted to fructose-6-phosphate by phosphoglucose isomerase (Chaudhry and Varacallo, 2026). This intermediate is subsequently phosphorylated to fructose-1,6-bisphosphate via phosphofructokinase (PFK), a key regulatory enzyme governing glycolytic potential (Chaudhry and Varacallo, 2026). Through a series of downstream reactions, phosphoenolpyruvate is ultimately converted to pyruvate by pyruvate kinase (Chaudhry and Varacallo, 2026), completing the glycolytic pathway. The metabolic fate of pyruvate represents a central position in cellular metabolism, linking glycolytic metabolism to oxidative metabolism.

Pyruvate Metabolism

Pyruvate, generated from glucose through glycolysis, contributes to a central regulatory position in skeletal muscle metabolism. It serves as a key branching point between glycolytic metabolism and oxidative metabolism that determines whether carbons are recycled, oxidized, or

conserved. Within skeletal muscle, pyruvate has two primary metabolic fates. It can be reduced to lactate by lactate dehydrogenase alpha (LDH α), a reaction that regenerates NAD⁺ from NADH to sustain glycolytic flux under conditions of high energetic demand (Farhana and Lappin, 2025). The lactate produced may be exported from muscle and transported to the liver, where it undergoes gluconeogenesis to regenerate glucose through the Cori cycle. This newly synthesized glucose can then re-enter circulation and return to muscle to support continued glycolysis, resulting in an important metabolic recycling system (Nalbandian et al., 2020; Farhana and Lappin, 2025). Alternatively, lactate can be oxidized back to pyruvate via lactate dehydrogenase beta (LDH β), thereby facilitating entry into mitochondrial oxidative pathways. Interestingly, downregulation of LDH β has been associated with high-feed efficient livestock, suggesting that altered lactate handling may contribute to differences in nutrient partitioning and metabolic efficiency (Fu et al., 2017).

When pyruvate is not reduced to lactate, it can be transported into the mitochondria, where it enters the tricarboxylic acid (TCA) cycle through two distinct enzymatic routes. The predominant pathway involves conversion of pyruvate to acetyl-CoA by the pyruvate dehydrogenase (PDH) complex (Patel et al., 2014). This enzyme complex catalyzes the oxidative decarboxylation of pyruvate, producing acetyl-CoA and NADH while releasing carbon dioxide (CO₂) (Patel et al., 2014). The activity of PDH is tightly regulated by pyruvate dehydrogenase kinases (PDKs) (Patel et al., 2014), which phosphorylate and inactivate PDH when cellular energy status is elevated. Among the PDK isoforms, PDK2 and PDK4 are the most abundantly expressed in skeletal muscle (Stellingwerff et al., 2003). Indeed, when PDK4 is upregulated, fatty acid oxidation is promoted and often forced while preserving glucose molecules (Sugden et al., 2000).

Collectively, the PDH and PDK regulatory interplay plays a critical role in determining whether pyruvate-derived carbon is oxidized for ATP production or redirected toward alternative pathways.

In contrast to PDH-mediated entry, pyruvate can also enter the TCA cycle through pyruvate carboxylase (PC), which catalyzes the ATP-dependent carboxylation of pyruvate to form oxaloacetate (OAA). This reaction requires biotin as a cofactor and Mg^{2+} as a coenzyme. Importantly, entry through PC conserves all three carbons of pyruvate, whereas entry through PDH results in the loss of one carbon as CO_2 (Minet and Gaster, 2010; Patel et al., 2014). From a nutrient efficiency perspective, this distinction is substantial, as carbon retained within the TCA cycle can contribute to anaplerosis and support biosynthetic pathways, including amino acid synthesis (Som et al., 2024; Fink et al., 2025). Indeed, PC has been shown to be upregulated in high feed-efficient pigs, whereas the PDH complex is downregulated in these animals (Fu et al., 2017), suggesting a metabolic shift favoring carbon retention over complete oxidative loss.

Collectively, the metabolic routing of pyruvate represents a pivotal determinant of nutrient partitioning within skeletal muscle. Whether carbon is recycled as lactate, oxidized through PDH with loss CO_2 , or conserved through PC-mediated anaplerosis the route of pyruvate has important implications for substrate efficiency and lean tissue accretion. Therefore, understanding the regulatory mechanisms governing pyruvate fate is essential for defining how skeletal muscle metabolism contributes to feed efficiency and production performance in livestock.

Mitochondrial Bioenergetics

Mitochondria serve as the central organelle of oxidative metabolism and play a critical role in determining how skeletal muscle utilizes nutrients (Albers et al., 2015; Rimmer et al., 2024). Following glycolysis, pyruvate generated in the sarcoplasm can be transported into mitochondria where it undergoes further metabolism to support ATP production. Within the mitochondrial

matrix, pyruvate enters the TCA cycle through PDH or PC (Minet and Gaster, 2010; Patel et al., 2014). The TCA cycle functions as a key metabolic pathway that undergoes a variety of oxidation steps utilizing carbons to generate reducing equivalents in the form of NADH and FADH₂, which subsequently donate electrons to the electron transport chain (ETC) located along the inner mitochondrial membrane. Electron transfer through the ETC drives proton pumping across the inner membrane, establishing an electrochemical gradient that powers ATP synthase to generate ATP through oxidative phosphorylation. Through this coordinated system of metabolic pathways, mitochondria integrate substrates derived from carbohydrates, lipids, and amino acids to support cellular energy production and metabolic homeostasis. Therefore, understanding mitochondrial function and how it plays a fundamental role in regulating skeletal muscle metabolism may ultimately influence animal growth performance and feed efficiency in livestock.

Mitochondrial Structure, Organization and Dynamics

Mitochondria are unique double-membrane organelles specialized to support oxidative metabolism and ATP synthesis. Although often oversimplified as the “powerhouse of the cell,” mitochondria perform functions that extend beyond energy production. The outer mitochondrial membrane contains proteins involved in nutrient import, metabolite export, and cellular signaling pathways (Moore and Ting, 2008; Mitchell, 2011; Wiedemann and Pfanner, 2017). In contrast, the inner mitochondrial membrane is highly impermeable and houses the complexes of the ETC as well as ATP synthase, which together drive oxidative phosphorylation (Rath et al., 2021). This inner membrane is extensively folded into structures known as cristae, which increase the surface area available for ETC components and enhance the capacity for ATP production (Hu et al., 2020). Indeed, cristae are highly dynamic structures that can rapidly undergo morphological changes in response to changes in cellular energy demand, physiological stress, and aging (Hu et al., 2020).

Between the outer and inner membranes lies the intermembrane space, where protons accumulate during electron transport to establish the electrochemical gradient that ultimately drives ATP synthesis. Within the mitochondrial matrix resides the TCA cycle and its associated intermediates. While the TCA cycle is primarily known for generating reducing equivalents that fuel the ETC, the cycle is not a carbon sinkhole. In fact, TCA cycle intermediates can leave the cycle through cataplerotic reactions or replenished through anaplerotic pathways depending on cellular metabolic demands (Bruce et al., 2001). Cataplerosis involves the export of TCA intermediates for use in biosynthetic pathways. For example, glutamic-oxaloacetic transaminase 2 catalyzes the conversion of oxaloacetate to aspartate through transamination. Aspartate can then be transported out of the mitochondria via the glutamate–aspartate antiporter and converted to glutamate, which may serve as a precursor for amino acid synthesis or be further catabolized to support ATP production (Cruzat and Tirapegul, 2015). Conversely, anaplerosis occurs when glutamate is converted into α -ketoglutarate in order to replenish the TCA cycle intermediate (Plaitakis et al., 2017). Overall, rather than functioning solely as refueling TCA intermediates, the TCA cycle operates as a highly dynamic metabolic network in which intermediate flux continuously adapts depending on cellular energy needs and biosynthetic requirements. In fact, carbon flux has been shown to vary across a pig's lifespan, depending on its developmental stage (Rimmer et al., 2024). Collectively, this highly organized structure enables mitochondria to efficiently couple substrate oxidation to ATP production and supports the metabolic demands of skeletal muscle. The dynamics of mitochondria within skeletal muscle are strongly influenced by muscle fiber type. Oxidative type I muscle fibers typically contain mitochondria arranged in dense, interconnected networks that support sustained oxidative metabolism, whereas glycolytic type II fibers often display a more fragmented and punctate mitochondrial morphology (Gerrard and Grant, 2003;

Chakkalakal et al., 2011). The coordinated balance between mitochondrial fusion and fission regulates mitochondrial quality, abundance, and metabolic capacity, enabling skeletal muscle to continually adapt mitochondrial function in response to nutrient availability, changing energetic demands and metabolic conditions.

The Tricarboxylic Acid Cycle

Mitochondria within skeletal muscle play a central role in nutrient metabolism. Pyruvate produced from glycolysis can enter mitochondria through the mitochondrial pyruvate carrier. Once pyruvate enters the mitochondrial matrix, it can either be converted to acetyl-CoA by PDH or carboxylated to oxaloacetate by PC (Minet and Gaster, 2010; Patel et al., 2014; Alabduladhem and Bordoni, 2026). Within the TCA cycle, the four-carbon oxaloacetate condenses with two-carbon acetyl-CoA to form the six-carbon intermediate citrate, a reaction catalyzed by citrate synthase (Alabduladhem and Bordoni, 2026). Citrate is subsequently converted to the five-carbon intermediate α -ketoglutarate, producing NADH and releasing one molecule of CO₂. Alpha-ketoglutarate is then converted to the four-carbon intermediate succinate, generating another molecule of CO₂. Succinate is oxidized to the four-carbon fumarate by succinate dehydrogenase while reducing flavin adenine dinucleotide (FAD) to FADH₂. Fumarate is subsequently converted to the four-carbon intermediate malate, which is then oxidized back to four-carbon oxaloacetate, completing the cycle (Alabduladhem and Bordoni, 2026). Overall, oxidation of one pyruvate molecule through the TCA cycle yields three NADH, one FADH₂, and one GTP, which provide reducing equivalents that support ATP generation through the electron transport chain (Alabduladhem and Bordoni, 2026).

Proper mitochondrial function relies on the efficient transport of pyruvate into the mitochondrial matrix to support TCA cycle flux and oxidative metabolism (Wiedemann and

Pfanner, 2017; Sharma et al., 2019). This process is mediated by the mitochondrial pyruvate carrier, which regulates the entry of glycolysis-derived pyruvate into the TCA cycle. Disruption of mitochondrial pyruvate carrier function can substantially alter skeletal muscle metabolism. For example, deletion of the mitochondrial pyruvate carrier in mice has been shown to inhibit fatty acid oxidation while promoting the Cori cycle, which contributed to increased energy expenditure and reduced adiposity while maintaining muscle mass and strength (Sharma et al., 2019). Additionally, reductions in TCA cycle intermediate availability can restrict oxidative metabolism and stimulate alternate anaplerotic pathways to replenish intermediates required to sustain metabolic flux. In fact, a downregulation of TCA cycle intermediates is a major driver of muscle atrophy (Fernando et al., 2023), highlighting the importance of maintaining the TCA cycle intermediate pool. In livestock species, shifts in mitochondrial substrate utilization may have important implications for production efficiency, as the balance between oxidative metabolism and alternative metabolic pathways ultimately influences how nutrients are partitioned between energy production, maintenance, and lean tissue accretion. In fact, nutrient utilization within the TCA cycle is dynamic as a pig grows (Rimmer et al., 2024), highlighting the importance of understanding mitochondrial function as a means to improve metabolic efficiency. Consequently, regulation of mitochondrial pyruvate transport and TCA cycle flux represents a critical control point governing skeletal muscle metabolic efficiency and overall animal performance.

Mitochondrial Respiration

Mitochondrial respiration is driven by the ETC, a series of protein complexes within the inner mitochondrial membrane that transfer electrons derived from reducing equivalents generated during glycolysis and TCA cycle (Balaban, 1990). The ETC is composed of five major complexes: NADH:ubiquinone oxidoreductase (Complex I), succinate dehydrogenase (Complex II),

coenzyme Q–cytochrome c reductase (Complex III), cytochrome c oxidase (Complex IV), and ATP synthase (Complex V) (Mitchell, 1961). Electrons carried by NADH and FADH₂ enter the ETC at Complexes I and II, respectively, and are sequentially transferred through Complexes III and IV. As electrons move through these complexes, protons are pumped from the mitochondrial matrix into the intermembrane space, generating an electrochemical gradient across the inner mitochondrial membrane. This proton motive force is subsequently utilized by ATP synthase to drive the phosphorylation of ADP to ATP termed oxidative phosphorylation.

The efficiency of oxidative phosphorylation can be evaluated through measurements of mitochondrial respiration, including basal respiration and maximal (uncoupled) respiration (Hong and Munoz-Canoves, 2023). Basal respiration reflects the oxygen consumption required to meet cellular ATP demand under physiological conditions (Hong and Munoz-Canoves, 2023), whereas uncoupled respiration represents the maximal respiratory capacity when the proton gradient is disrupted by chemical uncouplers (Mitchell, 1961; Fernando et al., 2023; Smolina et al., 2023). Importantly, not all protons re-enter the mitochondrial matrix through ATP synthase. A portion of protons instead return through proton leak, which reduces the efficiency of oxidative phosphorylation by protons re-entering the matrix without generating ATP (Mitchell, 1961; Smolina et al., 2023). In fact, proton leak has been shown to be inversely related to muscle mass across species (Porter and Brand, 1993). Additionally, mitochondrial oxygen consumption rates are altered in pig skeletal muscle undergoing increased hypertrophy compared with control pigs (Scheffler et al., 2014), suggesting that mitochondrial respiratory capacity contributes to skeletal muscle growth and metabolic efficiency. Collectively, the balance between basal respiration, maximal respiratory capacity, and proton leak provides valuable insight into mitochondrial

efficiency, ultimately influencing how effectively skeletal muscle converts and utilizes nutrient substrates.

Conclusion

Collectively, this literature review highlights skeletal muscle as a central regulator of nutrient utilization and growth efficiency in livestock species. Skeletal muscle metabolism contributes to carbohydrate availability, glycolytic flux, and mitochondrial metabolism to determine how nutrients are partitioned between cellular maintenance and muscle accretion. Mitochondrial function regulates the TCA cycle and oxidative phosphorylation. The balance between substrate oxidation, replenishment of TCA cycle intermediates, and mitochondrial respiratory efficiency ultimately shapes the metabolic phenotype of skeletal muscle. Consequently, shifts in mitochondrial substrate utilization, respiratory capacity, and proton leak may alter the metabolic efficiency of animals by how they convert dietary nutrients into lean tissue growth.

Although advances in understanding skeletal muscle metabolism has been investigated, the mechanisms by which skeletal muscle distribute and utilize nutrients remain incompletely defined. Therefore, further investigation into the metabolic pathways governing skeletal muscle metabolism is essential for identifying biological mechanisms that could contribute to improved production efficiency. The following studies aim to address these knowledge gaps by evaluating how naturally occurring genetic variations that influence skeletal muscle metabolism alter nutrient utilization and mitochondrial function. Understanding how these genetic models influence skeletal muscle metabolism will provide insight into the metabolic mechanisms that govern changes in growth performance and feed efficiency in livestock.

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Chapter 2 - Evaluating the biological and metabolic changes in myophosphorylase deficient skeletal muscle

Introduction

Skeletal muscle is a metabolically dynamic tissue that plays a central role in animal growth, production efficiency, and overall performance (Gerrard and Grant, 2003). In meat-producing animals, muscle metabolism directly influences how dietary nutrients are partitioned between lean tissue accretion and energy expenditure, ultimately impacting feed efficiency and carcass composition (Gerrard and Grant, 2003; England et al., 2014; Scheffler et al., 2014; Kirkpatrick et al., 2023; Rimmer et al., 2024). Although improvements in genetics and nutrition have markedly enhanced livestock productivity, mechanisms governing nutrient allocation between glycolytic and oxidative metabolic pathways within skeletal muscle remain incompletely understood. Defining how muscle adapts to alterations in substrate availability is therefore critical for advancing strategies that improve growth efficiency in agricultural systems.

Glycogen serves as a primary carbohydrate reserve in skeletal muscle and provides a rapid source of glucose to support ATP production during periods of increased energetic demand (Cori and Cori, 1940; Jensen et al., 2011). The mobilization of glycogen is catalyzed by muscle glycogen phosphorylase (myophosphorylase), which cleaves glycogen to generate glucose-1-phosphate for entry into glycolysis and downstream oxidative metabolism (Cori and Cori, 1940; Jensen et al., 2011). Through this process, myophosphorylase plays a key role in maintaining cellular energy balance, supporting contractile activity, and enabling metabolic flexibility in skeletal muscle (Cori and Cori, 1940; Chasiotis, 1983; Jensen et al., 2011; Stenvers et al., 2019; Almodovar-Paya et al., 2020). Disruption of this pathway fundamentally alters the capacity of muscle to generate ATP from carbohydrate substrates. Loss-of-function mutations in the myophosphorylase gene (*PYGM*)

result in an inability to degrade glycogen, a condition classically exemplified by McArdle disease in humans (Mc, 1951; Mellick et al., 1962; Almodovar-Paya et al., 2020). Individuals with McArdle disease accumulate excessive intramuscular glycogen and exhibit impaired glycolytic flux, leading to pronounced alterations in muscle energetics and mitochondrial function (Lam et al., 2025). These metabolic constraints necessitate compensatory reliance on alternative oxidative pathways to sustain ATP production. While this condition highlights the importance of glycogen metabolism in skeletal muscle, how the absence of glycogen utilization reshapes glycolytic and oxidative metabolism, mitochondrial regulation, and nutrient allocation at the skeletal muscle level remains poorly defined.

The naturally occurring *PYGM* mutation in cattle (referred to as PYGM cattle) provides a unique *in vivo* agricultural model to investigate skeletal muscle metabolism in the absence of functional glycogen breakdown. This model allows direct evaluation of how muscle adapts to chronic impairment of glycogenolysis under physiological growth conditions relevant to muscle growth and meat development. Understanding how skeletal muscle compensates for this metabolic limitation has important implications for animal growth efficiency, as shifts in substrate utilization and mitochondrial metabolism may influence lean tissue accretion and energetic efficiency (Fu et al., 2017; Kendall et al., 2026). Therefore, the objective of this study is to define the metabolic adaptations that occur in skeletal muscle of cattle harboring a naturally occurring mutation in the *PYGM* gene. Specifically, this work aims to evaluate alterations in glycolytic and oxidative metabolic pathways, mitochondrial enzyme abundance, and energy production capacity to better understand how impaired glycogen utilization influences muscle metabolic phenotype and nutrient allocation in a livestock production context.

Materials and Methods

Animal Harvest and Sample Collection

The protocol used in this experiment was approved by UNL IACUC Protocol Number: 2226. Using the genetic line from Batt et al. (2024), 3 PYGM deficient calves (referred to as PYGM cattle) were weaned and background from a Nebraska herd, as well as 3 age, weight, and composite (Red Angus x Simmental x Gelbvieh) matched calves from a separate Nebraska herd, were transported to UNL Eastern Nebraska Research, Extension and Education Center (ENREEC) feedlot (Mead, NE). Cattle were subjected to commercial feeding and finishing standards. The diet contained 51% high-moisture corn, 40% sweet bran (Cargill; Blair, NE), 5% corn stalks, and 4% supplement (DM basis), including monensin at 33.07 mg/kg (Rumensin; Elanco) and tylosin at 9.7 mg/kg on a DM basis (Tylan; Elanco). Following a 200 day feeding period, cattle were transported to the UNL Meat Loeffel Meat Lab, allowed a 12 hr lairage period and subjected to humane harvest and sampling of tissue. One PYGM deficient steer was deceased prior to transport. On the day of harvest, samples from the *longissimus dorsi* (LD), *latissimus dorsi* (LAT), *semitendinosus*-white portion (STW), *semitendinosus*-red portion (STR) and *masseter* (MS) were collected immediately after exsanguination. Samples were divided and snap frozen in liquid nitrogen, frozen for histology or used to isolate mitochondria. Snap frozen samples chopped into cubes, stored in whirlpak bags and frozen at -80 °C until further analysis. Histology samples were placed in plastic cryomolds containing Optimal Cutting Media (Fisher Scientific, Hampton, NH, USA), submerged in liquid nitrogen-cooled methyl butane and stored at -80 °C.

PYGM Variant Validation

To validate that the PYGM cattle lacked the PYGM protein, a Western blot was performed (Figure 2.1). Samples were prepped for Western blotting as described below, with minor

alterations. Following SDS-PAGE and membrane transfer onto a nitrocellulose membrane, the membrane was incubated with a 1:1,000 dilution of glycogen phosphorylase (anti-PYGM (Cat # NBP2-16689, Novus Biologicals, Centennial, CO, USA)) overnight at 4 °C. Blots were washed three times in 1x TBS-T and then incubated with IRDye® 800CW goat anti-rabbit IgG secondary antibody (LI-COR Biosciences, Lincoln, NE, USA) and images were taken under a fluorescence imager. To allow for normalization of total protein, blots were stained using Revert™ 700 Total Protein Stain (LI-COR Biosciences, Lincoln, NE, USA). All membranes were imaged on a LI-COR Odyssey® Infrared Imaging System, and band densities were quantified using Image Studio Lite Software. Relative abundance for PYGM is presented as arbitrary units (AU) normalized to total lane protein, as shown in Figure 2.1.

Western Blotting

Mitochondrial fraction samples were prepared through the differentiation and derivative protocol described by (Scheffler et al., 2014; Matarneh et al., 2021; Rimmer et al., 2024) to evaluate differences in mitochondrial enzymes compared to whole muscle samples using Western blotting procedures. Briefly, muscle (~40 g) was placed in ice-cold homogenization buffer (pH 7.4) containing 100mM sucrose, 180mM KCl, 50 mM tris-base, 5 mM MgCl₂, 1 mM K-ATP and 10 mM of 0.5M EDTA. Muscle was weighed and finely minced with scissors at a 15% (wt/vol) in homogenization buffer. Minced tissue was homogenized using a Potter-Elvehjem tissue homogenizer system (Glass-Col, Terre Haute, IN, USA), diluted with additional 20 mL of homogenization buffer and filtered through cheesecloth. The filtered solution was centrifuged using a Sorvall ST 40R centrifuge (Thermo Scientific, Waltham, MA, USA) at 2,000 ×g for 10 min at 4 °C. Samples were filtered through cheesecloth once more and centrifuged at 5,000 ×g for 20 min at 4 °C. Following centrifugation, the supernatant was discarded and the mitochondrial pellets

were resuspended in a modified KHEB buffer (pH 7.2) containing 120 mM KCl, 0.2 mM KH₂PO₄, 1 mM HEPES and 0.5 mM EGTA. Total protein quantification of the isolated mitochondria was assessed using the Thermo Scientific™ Pierce™ BCA Protein Assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Mitochondria was diluted to 3 µg/µL in titin buffer containing 0.05% bromophenol blue as described below.

Whole muscle samples were powdered using a liquid nitrogen-cooled mortar and pestle, and 100 mg of tissue was homogenized in cold titin buffer containing 8 M urea, 2 M thiourea, 3.5 M glycerol, 3% SDS (wt/vol), and 75 mM dithiothreitol using a bead homogenizer (BeadBlaster™ Homogenizer, Millipore Sigma, St. Louis, MO, USA). Samples were transferred to a clean microcentrifuge tube and centrifuged at 10,000 × g for 5 min. The supernatants were collected and heated at 60 °C for 10 min. Protein concentration was determined using the Bio Rad RC DC™ (reducing agent and detergent compatible) Protein Assay kit according to the manufacturer's instructions. Samples were diluted to 3 µg/µL with titin buffer containing 0.05% bromophenol blue.

Equal amounts of protein (30 µg) were separated using SDS-PAGE, transferred to PVDF or NC membranes, and blocked for one hour with OneBlock blocking buffer (Prometheus Protein Biology Products, Genesee Scientific, San Diego, CA, USA). Membranes were incubated with a primary antibody overnight on a rocker at 4 °C. Secondary antibody was added to the membranes and incubated for one hour prior to imaging. The secondary antibodies used included Goat anti-rabbit IgG (H + L) Highly Cross-Absorbed Secondary Antibody, Alexa Fluor™ Plus 647, Invitrogen™ (Cat # PIA32733, Fisher Scientific, Hampton, NH, USA) and goat anti-rabbit IgG (H + L) antibody HRP conjugate (Cat # AP307P, Sigma Aldrich, St. Louis, MO, USA). The enzymes used for Western blot analysis are described as follows: primary antibody, primary

antibody concentration, transfer membrane, secondary antibody, secondary antibody concentration, and image detection). Lactate dehydrogenase alpha (anti-LDH α (Cat # NBPI-48336, Novus Biologicals, Centennial, CO, USA), 1:1,000, NC, goat anti-rabbit Alexa Fluor™ Plus 647, 1:10,000, fluorescence); Lactate dehydrogenase beta (anti-LDH β (Cat # NBPI-31945, Novus Biologicals, Centennial, CO, USA), 1:1,000, NC, goat anti-rabbit Alexa Fluor™ Plus 647, 1:10,000, fluorescence); Pyruvate carboxylase (anti-PC (NBPI-49536SS, Novus Biologicals, Centennial, CO, USA), 1:800, PVDF, goat anti-rabbit IgG (H + L) antibody HRP conjugate, 1:9,000, chemiluminescence); Pyruvate dehydrogenase alpha 1 (anti-PDHA1 (Cat # AV48137, Sigma Aldrich, St. Louis, MO, USA), 1:900, PVDF, goat anti-rabbit IgG (H + L) antibody HRP conjugate, 1:9,000, chemiluminescence); Citrate synthase (anti-CS (Cat # TA308265, OriGene, Rockville, MD, USA), 1:1,000, PVDF, goat anti-rabbit IgG (H + L) antibody HRP conjugate, 1:10,000, chemiluminescence); Succinate dehydrogenase alpha (anti-SDHA (Cat # NBP2-93922, Novus Biologicals, Centennial, CO, USA), 1:1,000, NC, goat anti-rabbit Alexa Fluor™ Plus 647, 1:10,000, fluorescence); and mitochondrial ATP synthase F1 subunit beta (anti-ATP5F1B (Cat # DZ41668, Boster Bio, Pleasanton, CA, USA), 1:500, PVDF, goat anti-rabbit IgG (H + L) antibody HRP conjugate, 1:10,000, chemiluminescence). Prior to imaging, ProSignal Pico ECL reagent (Prometheus Protein Biology, Genesee Scientific, San Diego, CA, USA) was added at a ratio of 1:1 to the PVDF membranes, incubated for 45 sec, drained and imaged with chemiluminescence at a 15 sec exposure time. All membranes were imaged with the IBright Imaging System (Thermo Fisher Scientific, Waltham, MA, USA). After imaging the protein of interest, membranes were stained with Invitrogen™ No-Stain™ Protein Labeling Reagent (Cat # A44717, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Using the Universal

mode on the IBright imager, total protein images were captured. All images were analyzed using the Thermo Fisher IBright Analysis Software and normalized to the total protein abundance.

Metabolite Analysis

Glycogen, glucose, glucose-6-phosphate (G6P), and lactate concentrations were evaluated by using the 96-well plate enzymatic analytical methods assay described by (Hammelman et al., 2003; Kirkpatrick et al., 2023). Muscle glucose, G6P and lactate were measured from 100 mg of powdered tissue homogenized in 0.5M perchloric acid using a bead homogenizer (BeadBlaster™ Homogenzier, Millipore Sigma, St. Louis, MO, USA). Samples were incubated on ice for 15 min for complete lysis followed by centrifugation ($13,000 \times g$ at 4 °C for 10 min). The supernatant was transferred to a new microcentrifuge tube containing 2 M KOH to neutralize the reaction. Glycogen samples were similarly prepared with slight alterations. Briefly, samples were homogenized in 1.25 M hydrochloric acid, incubated at 90 °C for 2 hr, centrifuged and neutralized with 1.25 M KOH.

To assess glucose and G6P concentrations, samples were incubated with reaction buffer containing 6.25% triethanolamine, 12.5% 200 mM EDTA (pH 7.4), 3.5% 1 M MgCl₂, and 95% NADP of total volume and initial absorbance (OD₁) values were measured at 350 nm. Following the first read, 1.9 U glucose-6-phosphate dehydrogenase (G6PDH) (Cat # 10165875001, Sigma Aldrich, St. Louis, MO, USA) was added to all samples and incubated for 20 min before measuring OD₂. Samples were incubated for an additional 20 min with 0.95 U hexokinase (Cat # 11426362001, Sigma Aldrich, St. Louis, MO, USA) and 11 mg/mL Na-ATP. A final OD₃ was measured, and final metabolite concentrations were calculated. Glucose-6-phosphate was determined by OD₂ – OD₁ calculated using a standard curve. Glucose was determined by OD₃ – OD₁ and calculated using a standard curve.

To measure muscle lactate, samples were diluted 1:4 in a reaction buffer containing 0.125 M of 5M HCl, 473 mM glycine, 2.92 mM β -Nicotinamide adenine dinucleotide sodium salt, and 2.73% hydrazine. Samples were plated in triplicate in a 96-well plate and absorbance (OD_1) was measured at 340 nm. Each tube received 1.75 U lactate dehydrogenase (Cat# L2625, Sigma Aldrich, St. Louis, MO, USA), incubated for 90 min at room temperature and absorbance (OD_2) was measured. Lactate was determined by $OD_2 - OD_1$ and calculated using a standard curve.

Muscle glycogen concentration was determined by adding samples diluted 1:4 to 2 mL of reaction buffer containing 1.35% triethanolamine, 12.5% 200 mM EDTA (pH 7.4), 3.5% 1 M $MgCl_2$, and 95% NADP of total volume. Initial absorbance (OD_1) was measured at 350 nm. Samples were incubated with 35 μ L 1.9 U of G6PDH, 35 μ L 0.95 U hexokinase, and 70 μ L 11 mg/mL Na-ATP. Reactions were allowed to incubate at room temperature for 20 min before triplicate aliquots from the tube were placed on a 96-well plate and absorbance (OD_2) measured at 350 nm. Glycogen was determined by $OD_2 - OD_1$ and calculated using a standard curve. All metabolites are reported as μ mol/g of tissue.

Phosphofructokinase Activity

The glycolytic enzyme, phosphofructokinase was measured using the protocol described by (Feliu et al., 1976; England et al., 2014). Briefly, 100 mg of powdered muscle tissue was homogenized in 1 mL of 100 mM K_2HPO_4 (pH 7.4), placed in an ice bath for 15 min and centrifuged at $13,000 \times g$ at 4 °C for 10 min. The supernatant (40 μ L) was transferred to a new tube containing 1.8 mL of reaction buffer (120 mM MES, 3.2 mM $MgSO_4 \cdot 7H_2O$, 2 mM K-ATP, 1 mM NADH, and 3 mM fructose-6-phosphate (Cat# F3627, Sigma Aldrich, St. Louis, MO, USA). An enzyme solution containing 1.2 U glycerol-3-phosphate dehydrogenase (Cat# 10127752001, Sigma Aldrich, St. Louis, MO, USA), 0.12 U triosephosphate isomerase (Cat# T2391, Sigma

Aldrich, St. Louis, MO, USA), and 1 U aldolase (Cat# A2714, Sigma Aldrich, St. Louis, MO, USA) was added to each sample containing buffer. Samples were plated in triplicate on a 96-well plate, and absorbance (OD) was measured at 340 nm every minute for 11 min. Slopes of each reaction were obtained when R-squared (RSQ) values were greater than 0.99.

Statistical Analysis

The data were analyzed as a split plot design with genotype as the whole plot factor and muscle as the sub plot factor. Data were considered significant when $p \leq 0.05$ and considered trending when $p \leq 0.10$. Means were separated by pairwise comparisons. Main effects (genotype or muscle) are reported when the interaction was not significant (genotype $\times$ muscle). Data are presented as the least square means $\pm$ standard error. Western blot data were log transformed to normalize the residuals, and data are presented as the back-transformed values. All data were analyzed using R (version 4.2.1, Indianapolis, IN, USA).

Results

A genetic model was used to evaluate how skeletal muscle compensates for an impaired ability to breakdown glycogen. Cattle harboring a naturally occurring variant in the *PYGM* gene (PYGM cattle) were identified, and skeletal muscle was harvested from both affected and control animals. Within each genotype, four muscles representing distinct inherent metabolic phenotypes were collected and analyzed to characterize alterations in skeletal muscle metabolic properties associated with the inability to catabolize glycogen. Muscle samples were collected from the *longissimus dorsi* (LD, glycolytic muscle), *latissimus dorsi* (LAT, mixed muscle), the white portion of the *semitendinosus* (STW, mixed muscle), the red portion of the *semitendinosus* (STR, mixed muscle), and the *masseter* (MS, oxidative muscle). Abundance of PYGM protein was measured to confirm the absence of glycogen phosphorylase in PYGM cattle (Figure 2.1). PYGM

cattle showed no detectable PYGM protein (Figure 2.1A; $p < 0.05$). In contrast, among control cattle muscles, PYGM abundance was greatest in the STW muscle, followed by the STR, LAT, and LD, with the MS muscle displaying the lowest PYGM abundance (Figure 2.1A; $p < 0.05$).

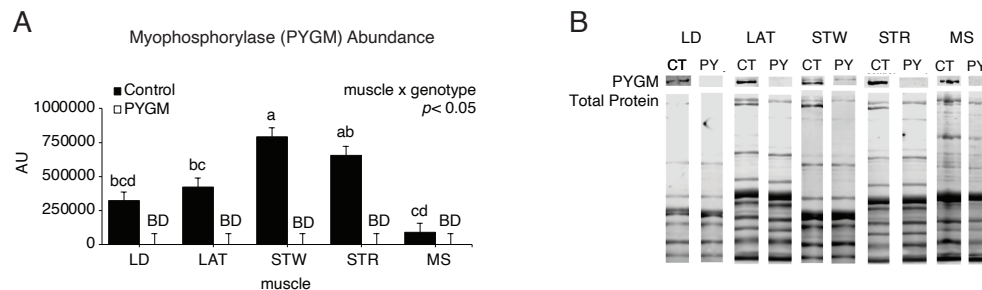


Figure 2.1. Abundance of myophosphorylase (PYGM) protein normalized to total protein in control and PYGM cattle. (A) Abundance presented as arbitrary units (AU) of myophosphorylase (PYGM). (B) Representative image of Western blot quantified in A. Data are shown as means $\pm$ SE. Two PYGM cattle ($n=2$) and three control cattle ($n=3$) per group. Means lacking a common letter (a, b, c, d) differ ($p \leq 0.05$). BD indicates below detection. CT indicates control cattle and PY indicates PYGM cattle.

Glycogen Metabolism and Glycolytic Metabolites

Key glycolytic metabolites and enzymes were assessed to investigate how skeletal muscle adapts to the inability to breakdown glycogen. Glycogen breakdown is initiated by myophosphorylase, which catalyzes the release of glucose-1-phosphate from glycogen. Glucose-1-phosphate is then converted back to glucose-6-phosphate (G6P), allowing entry into glycolysis and downstream ATP-generating pathways (Jensen et al., 2011; Patino and Orrick, 2025). The consumption of ATP results in the phosphorylation of glucose-1-phosphate to G6P by hexokinase. The following step in glycolysis is performed by the major regulatory enzyme phosphofructokinase (PFK), resulting in the production of fructose 1,6-bisphosphate. This essential enzyme is often used as an indicator of glycolytic flux. The final step in glycolysis is the

enzymatic conversion of phosphoenolpyruvate to pyruvate resulting in the production of ATP. Pyruvate has two fates once produced through glycolysis: conversion to lactate dehydrogenase through lactate dehydrogenase alpha (LDH α) and proceed into the Cori cycle where it can be converted back into glucose through gluconeogenesis in the liver or translocated from the cytosol into the mitochondrial matrix to fuel the tricarboxylic acid (TCA) cycle. Conversely, lactate can be converted back into pyruvate through lactate dehydrogenase beta (LDH β). The interplay between LDH α and LDH β acts as a metabolic switch that regulates energy production and is often used as an indicator of muscle adaption between glycolytic metabolism and oxidative metabolism (Liang et al., 2016; Farhana and Lappin, 2025). To evaluate how an impaired ability to breakdown glycogen influences glycolysis, concentrations of glycogen, glucose, G6P, and lactate were measured. Furthermore, protein abundance of LDH α and LDH β and PFK activity were evaluated to establish an understanding of glycolytic metabolism in the absence of glycogen breakdown.

Glycogen concentration was greatest in PYGM cattle compared to control cattle (Figure 2.2A; $p < 0.05$). Regardless of genotype, glycogen levels were highest in the STW and STR followed by LD, LAT and MS muscles (Figure 2.2B; $p < 0.05$). There were no differences in glucose levels for either genotype (Figure 2.2C; $p = 0.13$) or muscle (Figure 2.2D; $p = 0.39$). The concentration of G6P decreased in LAT, STW, and STR muscles from PYGM cattle compared to control cattle muscles (Figure 2.2E; $p < 0.05$). Lactate levels were highest in control STW and STR muscles followed by LAT, LD and MS muscles (Figure 2.2F; $p < 0.05$). No detectable lactate values were observed for PYGM cattle muscles (Figure 2.2F). Collectively, these findings indicate that impaired glycogen breakdown in PYGM cattle leads to altered glycolytic flux, characterized by glycogen accumulation, reduced downstream glycolytic intermediates, and an absence of lactate production across skeletal muscle types.

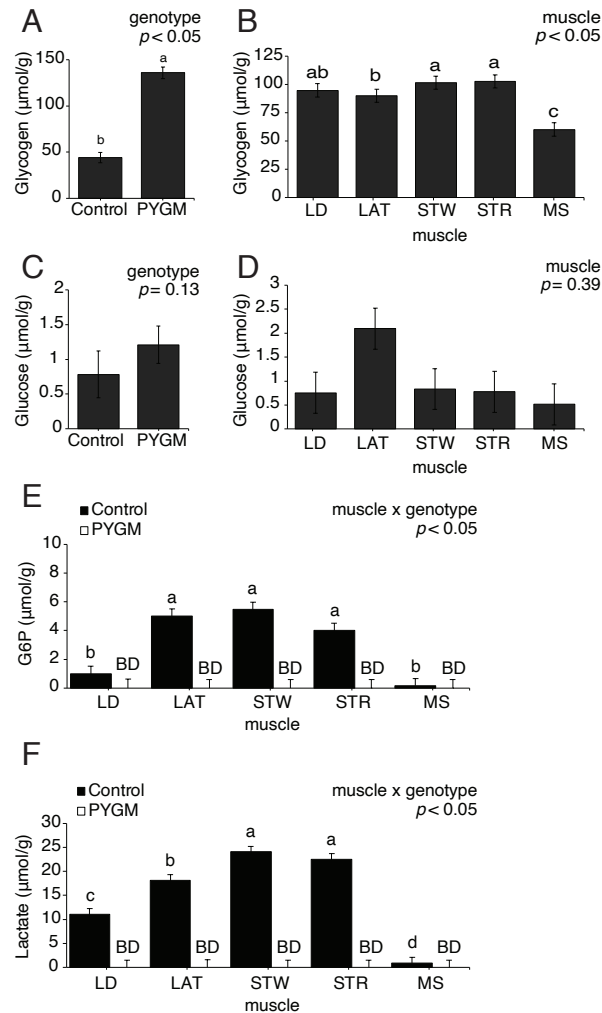


Figure 2.2. Glycolytic metabolite concentrations are altered in PYGM cattle. (A-B) Concentration of glycogen in each (A) genotype and (B) muscle. (C-D) Concentration of glucose in each (A) genotype and (B) muscle. (E) Concentration of glucose-6-phosphate (G6P) and (F) Concentration of lactate in each genotype and muscle. Data are shown as means $\pm$ SE. Two PYGM cattle ($n=2$) and three control cattle ($n=3$) per group. Means lacking a common letter (a, b, c) differ ($p \leq 0.05$).

To further evaluate glycolytic metabolism in PYGM cattle, key glycolytic enzymes were investigated. The activity of PFK did not differ between genotypes (Figure 2.3A; $p = 0.47$), but

differed across muscle. The activity of PFK was highest in LD and STW muscles compared the LAT and MS, whereas STR had intermediate values (Figure 2.3B; $p < 0.05$). Western blot data are reported as relative protein abundance in whole muscle homogenates and mitochondrial fractions in those associated mitochondrial to distinguish differences in the whole cell versus mitochondrial compositional changes. The abundance of LDH α from whole muscle was greatest in LD and MS muscles of PYGM cattle compared to corresponding LD and MS muscles of control cattle (Figure 2.3C; $p < 0.05$). Conversely, LDH α from the mitochondrial fraction was highest in LD followed by STW, LAT, STR and MS (Figure 2.3E; $p < 0.05$), whereas genotype did not differ in abundance (Figure 2.3D; $p < 0.05$). Regardless of genotype in whole muscle homogenates, LDH β was greatest in LD, STW, and STR and lowest in LAT and MS (Figure 2.3G; $p < 0.05$). Mitochondrial fraction LDH β was highest in PYGM cattle compared to control cattle (Figure 2.3H; $p < 0.05$). Additionally, STR increased in LDH β compared to LAT, STW and MS with LD values being highest compared to LAT and MS (Figure 2.3I; $p < 0.05$). Together, these data indicate that glycolytic activity and enzyme abundance varied primarily by muscle, while genotype differences were observed in LDH α whole muscle and LDH β mitochondrial fraction preparations.

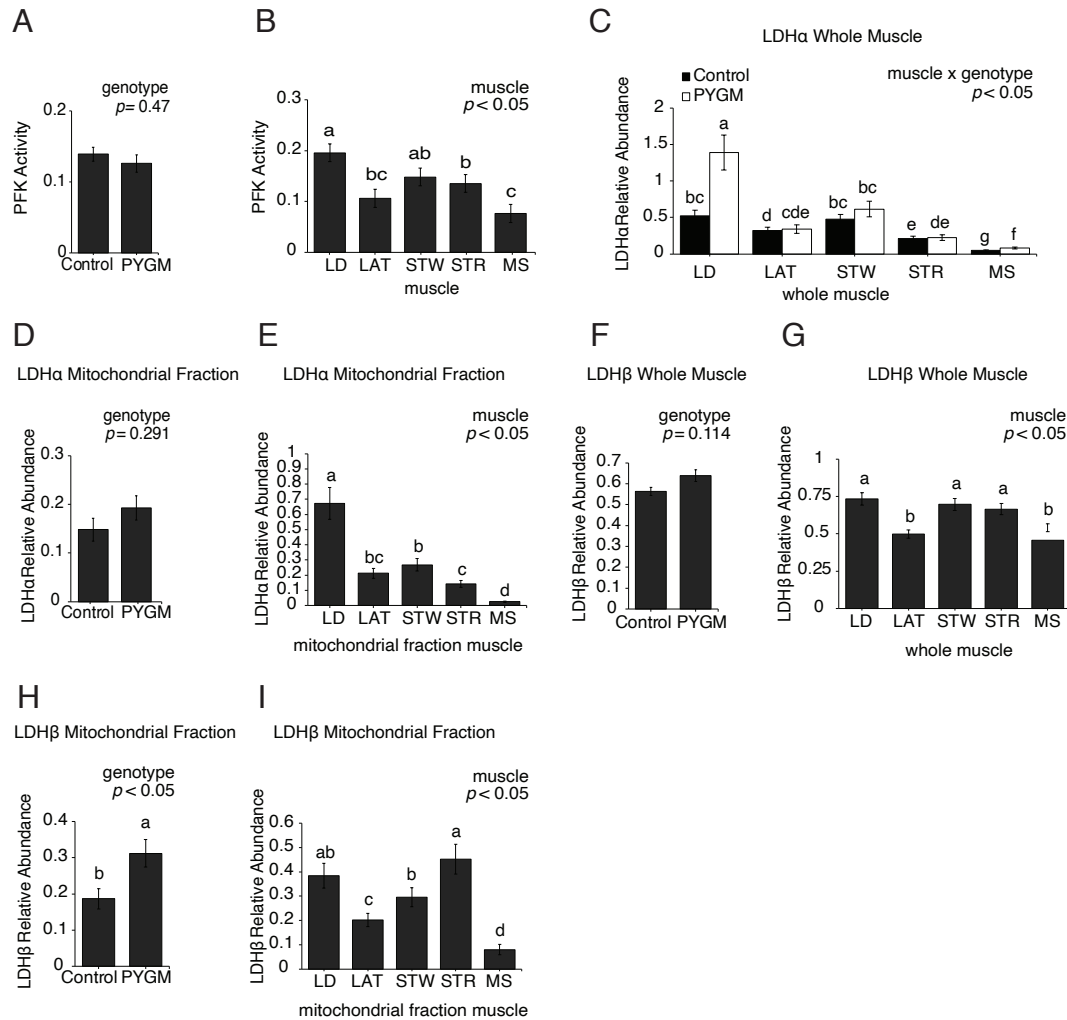


Figure 2.3. Enzymes indicative of glycolytic metabolism in whole muscle and mitochondrial fraction preparations. (A-B) Activity of phosphofructokinase (PFK) in each (A) genotype and (B) muscle. (C-E) Relative abundance of lactate dehydrogenase alpha (LDH α) in each (A) muscle and genotype using whole muscle samples and (D-E) mitochondrial fraction samples in each (D) genotype and (E) muscle. (F-G) Relative abundance of lactate dehydrogenase beta (LDH β) in (F-G) whole muscle samples in each (F) genotype and (G) muscle and (H-I) mitochondrial fraction samples in each (H) genotype and (I) muscle. Data are shown as means $\pm$ SE. Two PYGM cattle ($n=2$) and three control cattle ($n=3$) per group. Means lacking a common letter (a, b, c) differ ($p \leq 0.05$).

Pyruvate Entry into the Tricarboxylic Acid Cycle in Skeletal Muscle Mitochondria

Pyruvate, the end-product of glycolysis, plays a significant role in nutrient allocation connecting glycolysis to oxidative phosphorylation. Pyruvate can enter the TCA cycle through oxidation to acetyl-CoA by pyruvate dehydrogenase (PDH) or through pyruvate carboxylase (PC), a carbon-conserving anaplerotic pathway. The PDH complex is responsible for a majority of pyruvate entry into the TCA cycle (Patel et al., 2014); however, this catalytic step produces a carbon dioxide molecule, which is deemed wasted in terms of supporting muscle growth. Conversely, the carboxylation of pyruvate to oxaloacetate (OAA) by PC results in the preservation of carbon. The tight interplay between PDH and PC impacts nutrient allocation and carbon preservation within mitochondria. To investigate whether an impairment in glycogen breakdown changes how pyruvate enters into the TCA cycle, PDH α -1 and PC were evaluated. Abundance of PDH α -1 did not differ between genotypes in either whole muscle homogenates (Figure 2.4A; $p < 0.05$) or mitochondrial fraction (Figure 2.4C; $p < 0.05$). Whole muscle MS was greatest in PDH α -1 abundance compared to LA, STW, and STR with LAT having intermediate values (Figure 2.4B; $p < 0.05$). Similarly, mitochondrial fraction MS was highest compared to LD, STW and STR with LAT not differing among all muscles (Figure 2.4D; $p < 0.05$). Whole muscle PC increased in PYGM cattle LD and LAT muscles compared to their control muscles (Figure 2.4E; $p < 0.05$). Although, mitochondrial fraction PC was highest in STW followed by LD, STR, MS and lowest in LAT muscles (Figure 2.4G; $p < 0.05$), there were not differences between genotypes (Figure 2.4F; $p < 0.05$). Collectively, these data indicate impaired glycogen breakdown does not alter PDH abundance but is associated with muscle-specific changes in PC abundance.

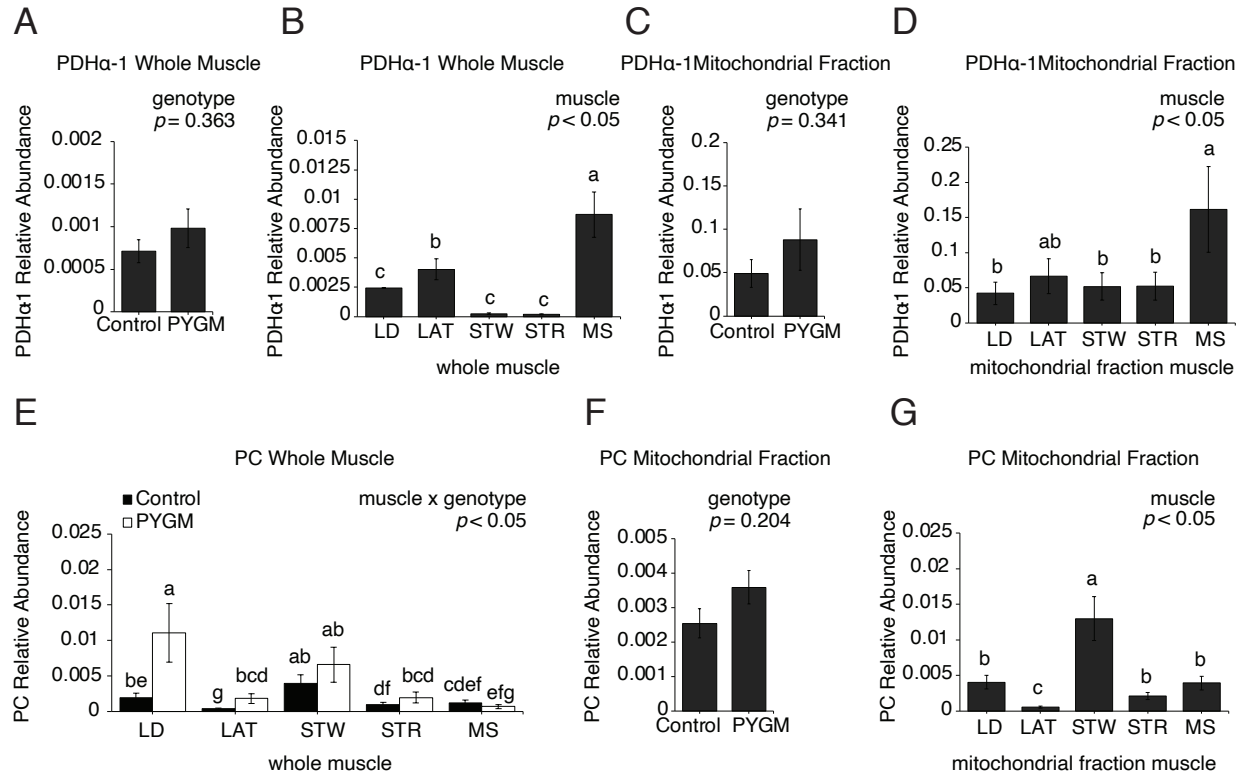


Figure 2.4. Enzymes indicative of pyruvate metabolism in whole muscle and mitochondrial fraction preparations. (A-D) Relative abundance of pyruvate dehydrogenase alpha- 1 (PDHα-1) in each (A-B) whole muscle samples in (A) genotype and (B) muscle. (C-D) Mitochondrial fraction muscle in each (C) genotype and (D) muscle. (E-G) Relative abundance of pyruvate carboxylase (PC) in (E) muscle and genotype whole muscle samples and (F-G) mitochondrial fraction samples in each (F) genotype and (G) muscle. Data are shown as means $\pm$ SE. Two PYGM cattle (n=2) and three control cattle (n=3) per group. Means lacking a common letter (a, b, c) differ ($p \leq 0.05$).

Abundance of Tricarboxylic Acid Enzymes Differs Based on Inherent Muscle Metabolism

To evaluate whether the inability to breakdown glycogen alters mitochondrial TCA cycle enzymes, citrate synthase (CS) and succinate dehydrogenase alpha (SDHα) were assessed. The enzyme CS was chosen as it is a key rate-limiting enzyme catalyzing the first step in the TCA cycle resulting in the condensation of acetyl-CoA and OAA to form citrate. Additionally, SDHα

catalyzes the conversion of succinate to fumarate reducing FAD to FADH₂, which are subsequently oxidized by complex II of the electron transport chain (ETC). Abundance of CS did not differ between genotypes in either whole muscle homogenates (Figure 2.5A; $p < 0.05$) or mitochondrial fraction samples (Figure 2.5C; $p < 0.05$). Whole muscle CS increased in the MS compared to STW and STR with LD and LAT showing intermediate levels (Figure 2.5B; $p < 0.05$). Mitochondrial fraction CS increased in MS compared to all other muscle types (Figure 2.5D; $p < 0.05$). Abundance of whole muscle SDH α increased in PYGM cattle compared to control regardless of muscle type (Figure 2.5E; $p < 0.05$). Whole muscle CS was highest in the LD compared to the LAT with STW, STR and MS having intermediate values (Figure 2.5F; $p < 0.05$). Conversely, mitochondrial fraction SDH α did not differ between genotypes (Figure 2.5G; $p < 0.05$). Across muscles SDH α was the highest in LD compared to STR with LAT, STW and MS (Figure 2.5H; $p < 0.05$). Overall, these results demonstrate disruptions in glycogen metabolism do not uniformly impact TCA cycle enzyme abundance within mitochondria, but may selectively influence enzymes linking the TCA cycle to the ETC.

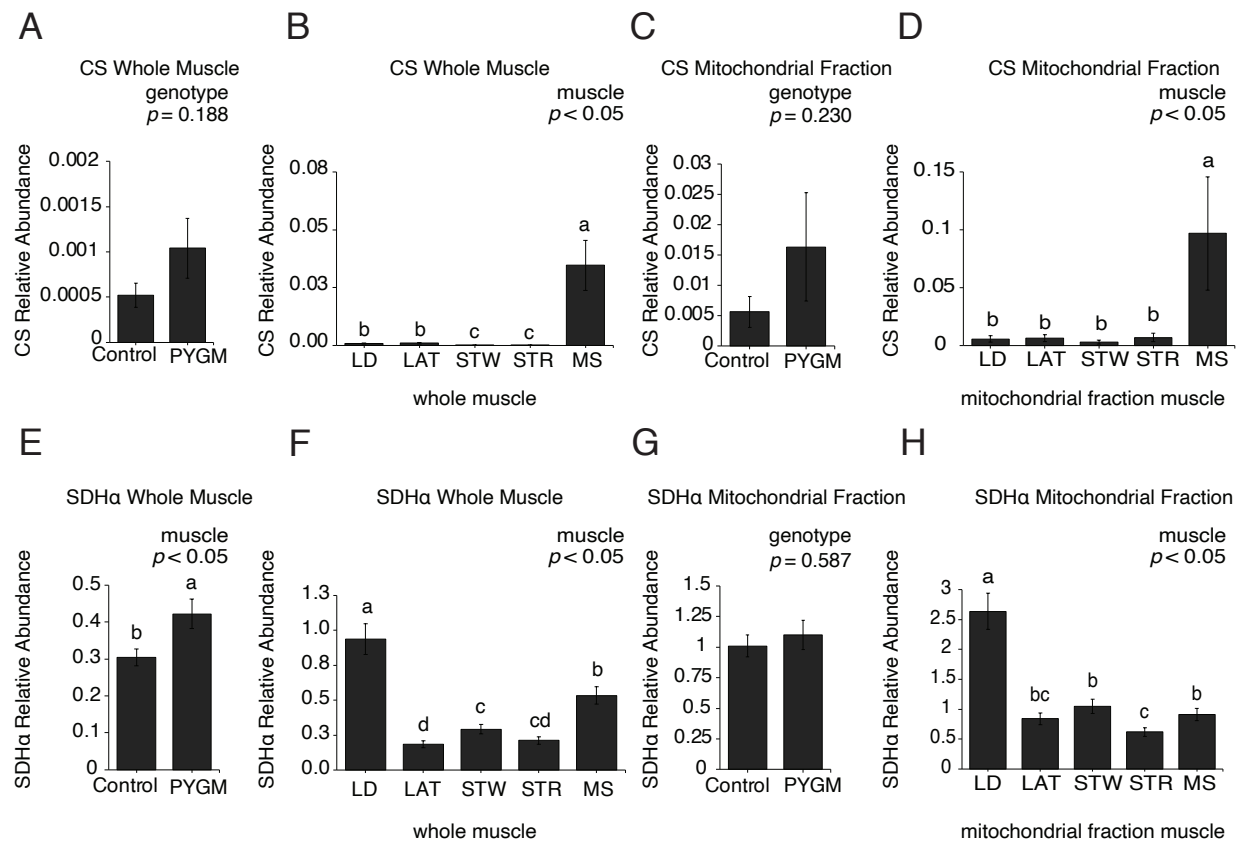


Figure 2.5. Enzymes indicative of oxidative metabolism. (A-D) Relative abundance of citrate synthase (CS) in (A-B) whole muscle samples and (C-D) mitochondrial fraction samples in each (A, C, E, G) genotype and (B, D, F, H) muscle. Data are shown as means $\pm$ SE. Two PYGM cattle ($n=2$) and three control cattle ($n=3$) per group. Means lacking a common letter (a, b, c) differ ($p \leq 0.05$).

Mitochondrial Energy Metabolism is Altered in Muscle with a PYGM Mutation

To identify changes in mitochondrial ATP production and overall mitochondrial activity in muscle harboring a naturally occurring mutation in the *PYGM* gene, the abundance of ATP synthase subunit beta (ATP5 β) was evaluated. The catalytic beta subunit is responsible for producing ATP from ADP and inorganic phosphate and is often used as an indicator for

mitochondrial energy production (Xu et al., 2013). Abundance of whole muscle ATP5 β increased in the LD, STW and STR muscles of PYGM cattle compared to control corresponding muscles (Figure 2.6A; $p < 0.05$). Alternatively, mitochondrial fraction ATP5 β increased in the MS of PYGM cattle compared to control MS muscles (Figure 2.6B; $p < 0.05$). Collectively, these results demonstrate that ATP5 β abundance was increased in several muscles of PYGM cattle, with differences observed in both whole muscle homogenates and mitochondrial fractions.

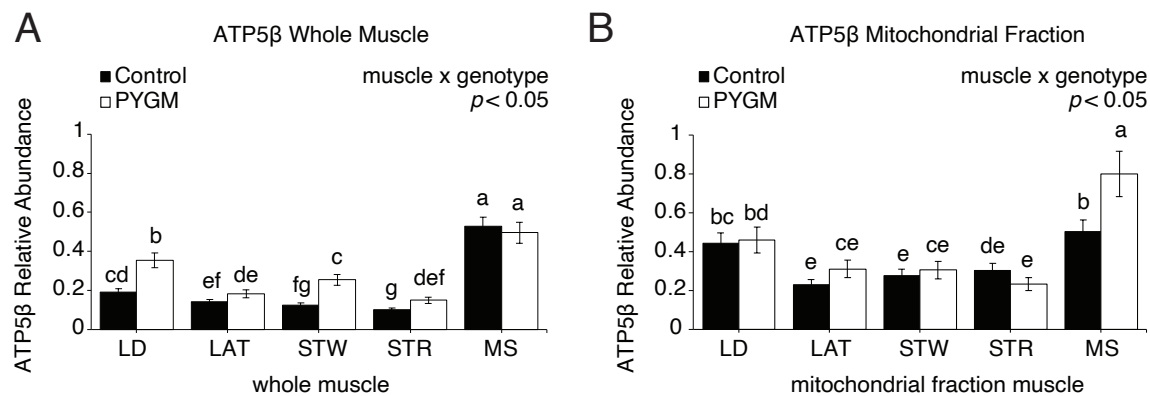


Figure 2.6. Mitochondrial ATP is altered by muscle with impaired ability to breakdown glycogen. (A-B) Relative abundance of mitochondrial ATP synthase subunit beta (ATP5 β) in (A) whole muscle samples and (B) mitochondrial fraction muscle samples. Data are shown as means $\pm$ SE. Two PYGM cattle (n=2) and three control cattle (n=3) per group. Means lacking a common letter (a, b, c) differ ($p \leq 0.05$).

Discussion

Skeletal muscle glycogen serves as a critical carbohydrate energy reserve that supports muscle metabolic flexibility, adaptability and ATP production as an animal grows (McVeigh and Tarrant, 1982; Gaster et al., 2001; Zhao et al., 2019). However, glycogen metabolism is highly dynamic and influenced by numerous physiological and nutritional factors. For instance, high-yielding dairy cows in early lactation exhibit reductions in muscle glycogen stores as nutrients are

redirected to support milk production (Kuhla et al., 2011). Similarly, dietary interventions have been shown to alter glycogen metabolism in beef cattle, where animals fed barley or hay displayed distinct changes in glycogen characteristics within the *M. longissimus dorsi* (McVeigh and Tarrant, 1982). Collectively, these observations highlight that glycogen metabolism is not static, but rather responsive to metabolic state, nutrient availability, and production demands.

Despite current literature identifying the importance of glycogen as an energy substrate, the mechanisms by which skeletal muscle adapts to impaired glycogen breakdown remain poorly defined. Therefore, this study presents a unique model using cattle harboring a naturally occurring *PYGM* variant, termed PYGM cattle, to evaluate how skeletal muscle compensates for the inability to effectively mobilize glycogen. Our data validate this model by evaluating glycogen myophosphorylase abundance (Figure 2.1) and glycogen concentrations (Figure 2.2). As expected, PYGM protein abundance was absent in PYGM cattle relative to control cattle ($p < 0.05$). Additionally, elevated glycogen concentrations, a hallmark characteristic of this mutation (Brull et al., 2015), was confirmed by our metabolite data demonstrating significantly greater glycogen levels in PYGM cattle compared to control cattle ($p < 0.05$). These findings are consistent with previous reports in Red Angus cattle carrying the same *PYGM* variant, where increased glycogen accumulation and a deficiency in PYGM protein were observed (Batt et al., 2024).

In addition to glycogen levels, glycolytic metabolites (glucose, G6P, and lactate) were measured to further evaluate how impaired glycogen breakdown influences glycolytic flux. Glucose concentrations did not differ between genotypes ($p = 0.13$) or muscle groups ($p = 0.39$), suggesting that basal glucose availability within skeletal muscle is likely maintained through uptake from circulating substrates derived from dietary carbohydrates. This observation aligns with findings reported in patients with McArdle disease, where resting glucose levels do not differ

from those of healthy individuals (Nielsen et al., 2002). Interestingly, G6P concentrations were greater in control LAT, STW, and STR muscles compared to PYGM cattle ($p < 0.05$), which suggests PYGM cattle have a reduced accumulation of G6P. The activity of PFK, a key regulatory enzyme of glycolysis catalyzing the irreversible conversion of fructose-6-phosphate to fructose-1,6-bisphosphate, is commonly used as an indicator of glycolytic capacity. No differences in PFK activity were detected between genotypes ($p = 0.47$); however, significant differences were observed among muscles. Consistent with their inherent metabolic characteristics, the glycolytic LD and STW muscles exhibited greater PFK activity compared to the oxidative MS muscle ($p < 0.05$). These data indicate PYGM cattle could be shunting metabolites towards the pentose phosphate pathway or hexosamine pathway, rather than proceeding through glycolysis. Further, lactate concentrations were elevated in control LD, LAT, STW, and STR muscles relative to PYGM cattle ($p < 0.05$). These findings suggest that the impaired glycogen breakdown does not alter the regulation of the early steps of glycolysis. In contrast, clear genotype differences were observed for downstream glycolytic intermediates.

Pyruvate, the end product of glycolysis and a central metabolite connecting glycolysis to mitochondrial oxidation, plays a pivotal role in dictating nutrient usage. The conversion of pyruvate to lactate is primarily mediated by LDH α , after which lactate may be exported from skeletal muscle and shuttled to the liver for gluconeogenesis via the Cori Cycle. The resulting glucose can then be recycled back into glycolysis. Conversely, lactate can be oxidized by LDH β to regenerate a process commonly associated with increased oxidative capacity (Elustondo et al., 2013). The interplay between LDH α and LDH β is often interpreted as an indicator of metabolic shifts between glycolytic and oxidative metabolism (Liang et al., 2016; Farhana and Lappin, 2025). The abundance of whole muscle LDH α increased in the LD and MS muscles from PYGM cattle

compared to the LD and MS muscles from control cattle ($p < 0.05$), whereas mitochondrial fraction LDH α did not differ between genotypes ($p = 0.291$). These findings are consistent with reports in McArdle disease patients, where LDH levels are elevated (Leite et al., 2012). These data suggest that PYGM cattle muscles adapt to the PYGM mutation by increasing the abundance of sarcoplasmic LDH α , which aligns with the reduced lactate concentrations observed in the metabolite data. Conversely, no differences were observed between genotypes in whole muscle LDH β ($p = 0.114$); however, the abundance of LDH β was highest in PYGM cattle compared to control mitochondrial fraction muscles ($p < 0.05$). These findings indicate that mitochondrial LDH β may play a role in driving metabolic adaptation, promoting enhanced oxidative capacity and improved energetic efficiency to offset the diminished glycolytic capacity in PYGM cattle (Liang et al., 2016). Together, these results demonstrate that PYGM cattle exhibit distinct differences in LDH isoforms, capturing the adaptive shift in lactate and pyruvate metabolism that may contribute to altered substrate utilization in PYGM variant muscle.

When pyruvate is not reduced to lactate, it may be transported into the mitochondrial matrix for oxidation within the TCA cycle. Pyruvate can enter the TCA cycle through the enzymes PDH or PC. The enzyme PDH catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA, resulting in carbon loss, whereas PC catalyzes the carboxylation of pyruvate to OAA. Entry by PC replenishes the TCA intermediate OAA while conserving carbon skeletons. Preservation of these carbon substrates supports anabolic processes, including amino acid synthesis (Prochownik and Wang, 2021). Consequently, changes in PDH and PC activity serve as key regulatory points governing nutrient partitioning, carbon retention, and overall metabolic efficiency. Despite this, the mechanisms by which skeletal muscle prioritizes pyruvate flux through PDH or PC remain largely understood. Although no genotype differences were detected for PDH α -1 abundance in

either whole muscle or mitochondrial fractions, PC abundance was greater in the LD and LAT whole muscles of PYGM cattle compared with the corresponding control muscles ($p < 0.05$). These findings suggest that inherently glycolytic muscles in PYGM cattle may alter nutrient partitioning by favoring pyruvate entry through PC. Within the TCA cycle, CS abundance did not differ between genotypes in either whole muscle ($p = 0.188$) or mitochondrial fractions ($p = 0.230$). In contrast, SDH α abundance was greater in PYGM cattle relative to control cattle ($p < 0.05$). This pattern may indicate that control cattle preferentially divert nutrients out of the TCA cycle and towards biosynthetic pathways prior to SDH α , whereas PYGM cattle exhibit increased SDH α abundance consistent with enhanced FADH $_2$ production to support cellular energy demands (Roudbari et al., 2019). This adaption aligns with the greater reliance of PYGM cattle on fatty acid oxidation (Leite et al., 2012; Lam et al., 2025). Notably, the energetic cost of synthesizing glucose (~17 kJ/g) is substantially lower than that required to generate acetyl-CoA from dietary fatty acids (~39 kJ/g) (Schonfeld and Reiser, 2013; Rauw et al., 2025). Together, these data suggest that muscle unable to breakdown glycogen may influence pathways regulating pyruvate fate and oxidative metabolism.

To further evaluate genotype differences in oxidative metabolism, the abundance of ATP5 β , a key component of mitochondrial ATP synthesis, was assessed. Interestingly, the abundance of ATP5 β was dependent upon genotype and muscle. Whole muscle ATP5 β increased in the LD, STW and STR muscles from PYGM cattle ($p < 0.05$); whereas, mitochondrial fraction ATP5 β increased in the MS from PYGM cattle compared to the MS from control cattle ($p < 0.05$). These data indicate that genotype influences muscle-specific components of oxidative phosphorylation, reflecting potential differences in energy demand. Additionally, the increase in ATP5 β may also be linked to upstream alterations in mitochondrial metabolism. In the present

study, SDH α abundance was elevated in PYGM cattle ($p < 0.05$), which could indicate enhanced conversion of succinate to fumarate resulting in a greater supply of reducing equivalents to the electron transport chain and may contribute to the elevated abundance of ATP β observed in these muscles (Ahmad et al., 2026).

Interestingly, muscles that exhibited increased ATP5 β in whole-muscle preparations (LD, STW, and STR) are primarily glycolytic or mixed in metabolic phenotype. In humans with McArdle disease, glycolytic muscles fatigue rapidly due to impaired glycogen breakdown (Brull et al., 2015). Therefore, the increased abundance of ATP5 β in these muscles may represent a compensatory response to support ATP production in muscles experiencing energetic stress. Consistent with this concept, patients with McArdle disease often exhibit impaired oxidative capacity (Villarreal-Salazar et al., 2022). However, in the present study, glycolytic and mixed muscles displayed increased ATP5 β abundance, suggesting that PYGM cattle may attempt to enhance mitochondrial energy production to offset limitations in glycogenolysis. Additionally, impaired oxidative capacity associated with McArdle disease has largely been demonstrated during exercise physiology experiments (Mellick et al., 1962; Villarreal-Salazar et al., 2022), whereas the present study evaluated skeletal muscle from cattle under normal physiological conditions. Further, despite impaired glycogen breakdown, no differences in glucose metabolites were detected between control and PYGM cattle ($p > 0.05$), suggesting that glucose consumption may be maintained or potentially increased in PYGM cattle to support ATP generation. Supporting this concept, PYGM cattle exhibited increased abundance of PC ($p < 0.05$), which may facilitate anaplerotic replenishment of OAA within the TCA cycle. This pathway could enhance mitochondrial metabolism while conserving carbon skeletons for biosynthetic processes, potentially improving metabolic efficiency despite impaired glycogen utilization. Finally,

individuals with McArdle disease often rely more heavily on fatty acid oxidation rather than glucose oxidation to sustain energy production (Rodriguez-Lopez et al., 2023). Therefore, the increased ATP5 β abundance observed in PYGM cattle may also reflect a metabolic shift toward greater reliance on alternative oxidative substrates, such as fatty acids, to support mitochondrial ATP generation in the absence of efficient glycogen breakdown.

Conclusion

Collectively, these findings demonstrate that skeletal muscle exhibits metabolic adaptability in response to impaired glycogen breakdown. Despite the absence of myophosphorylase and accumulation of glycogen, PYGM cattle muscle appear to maintain regulation of the beginning of glycolysis while displaying distinct alterations in downstream metabolic pathways. The observed shifts in glycolytic intermediates, LDH isoform abundance, and enzymes governing pyruvate fate suggest potential metabolic remodeling aimed to sustain cellular energy balance. Increased PC, SDH α , and ATP5 β abundance further support the concept that PYGM-deficient muscle may alter nutrient partitioning through oxidative phosphorylation to compensate for reduced glycolytic substrate utilization. Most notably, these data highlight that the inability to breakdown glycogen results in downstream compensatory adaptations in nutrient partitioning. The *PYGM* cattle model provides a unique *in vivo* system for investigating how skeletal muscle adapts its metabolic pathways under constraints in glycogen utilization. Together, this work sheds light on the dynamic response of muscle in response to the impaired ability of glycogen breakdown.

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Chapter 3 - Exploring the phenotypic and metabolic response of porcine skeletal muscle harboring mutations that drive oxidative and hypertrophic phenotypes

Introduction

Global population growth and the decline of arable land continue to impose unprecedented demands on agricultural systems, requiring more efficient livestock production with fewer resources. As developing nations experience economic growth, rising disposable income drives higher consumption of animal protein, further intensifying the global demand for meat. Currently, the U.S. pork industry produces approximately 27.6 billion pounds of pork annually (U.S. Department of Agriculture, 2025), and to meet projected demand, production is expected to increase by an additional 9.5 billion pounds by 2034 (Dohlman et al., 2025). To accommodate this growth, the swine industry has implemented strategies including genetic selection, improved nutrition, growth promotants, and feed additives (Wu et al., 2011; Khanal et al., 2019; Rao et al., 2023). While these approaches have greatly enhanced productivity, continued innovation is essential to further improve efficiency and sustainability under increasingly limited resource conditions.

A central area for improvement lies in feed conversion efficiency and a deeper understanding of nutrient utilization within the animal. For example, early-growing pigs currently exhibit feed to gain ratios (F/G) near 2:1, whereas finishing pigs require more than 3:1 F/G to achieve similar gains (Stender, 2012), highlighting metabolic inefficiencies that emerge during later growth phases. Enhancing nutrient partitioning and metabolic utilization has the potential to accelerate growth rates or increase lean tissue deposition, thereby improving overall production efficiency.

Understanding the biochemical mechanisms that regulate nutrient utilization within muscle is critical to improving swine growth efficiency. Skeletal muscle comprises 40–50% of an animal's body mass, utilizes up to 60-80% of dietary glucose, and plays a central role in nutrient metabolism and growth efficiency (Lee et al., 2010; Picard et al., 2010). Skeletal muscle is a metabolically diverse tissue consisting of fibers that range from oxidative to glycolytic profiles (Gerrard and Grant, 2003). Type I fibers are slow-contracting and rely primarily on oxidative metabolism to sustain prolonged activity, while Type II fibers (IIa, IIx, and IIb) are fast-contracting and vary in their metabolic reliance, with Type IIb being the most glycolytic. Type II fibers generally undergo greater hypertrophy and exhibit larger cross-sectional areas compared to Type I fibers (Gerrard and Grant, 2003). Interestingly, Scheffler et al. (2014) demonstrated that fiber hypertrophy and oxidative capacity can increase simultaneously, particularly in pigs harboring distinct genetic mutations affecting energy metabolism (RyR1^{R615C} and AMPK γ ^{R200Q}).

The ryanodine receptor 1 (*RYR1*) gene encodes a calcium (Ca²⁺) release channel located in the sarcoplasmic reticulum membrane of skeletal muscle and is essential for maintaining Ca²⁺ homeostasis. The porcine R615C mutation in *RYR1* (RyR1^{R615C}) leads to uncontrolled channel opening and constant Ca²⁺ release, disrupting cellular Ca²⁺ balance and shifting metabolism toward glycolytic pathways (Scheffler et al., 2014; Lawal et al., 2020). Conversely, the AMP-activated protein kinase (AMPK) complex functions as a cellular energy sensor that detects AMP/ADP levels and activates signaling cascades to restore ATP balance. The R200Q mutation in the *PRKAG3* gene (AMPK γ ^{R200Q}) impacts the AMPK γ 3 subunit resulting in the constitutive activation of AMPK, increasing mitochondrial content and oxidative capacity in skeletal muscle (Mahlapuu et al., 2004; Yan et al., 2021). When pigs harboring these mutations are crossed, the resulting RyR1^{R615C}(heterozygous) + AMPK γ ^{R200Q} (heterozygous) littermates display enhanced oxidative

capacity and greater muscle fiber hypertrophy at market weight (Scheffler et al., 2014). Although it is apparent that these mutations influence metabolic characteristics of skeletal muscle at market weight, the function of these mutations may be altered by the complex dynamics of postnatal muscle growth throughout a pig's life.

Postnatal growth can be broadly divided into four distinct phases. Although this framework represents a simplification of a highly complex biological process, it provides a useful conceptual model for illustrating the dynamic shifts in nutrient partitioning to tissue types throughout postnatal development. Following birth, phase I emphasizes the development of vital organs, the nervous system, visceral adipose tissue, and the skeletal framework. Phase II is characterized by rapid muscle tissue accretion, whereas phase III continues to increase muscle growth but at a steady pace. Finally, phase IV is primarily associated with increased fat deposition. Interestingly, a past study demonstrated that skeletal muscle is metabolically dynamic during porcine postnatal growth, and documented a period of robust muscle hypertrophy beginning around 87-120 days of age (Rimmer et al., 2024). Pigs carrying naturally occurring RyR1^{R615C} and AMPK γ ^{R200Q} mutations result in distinct differences at market weight (end of Phase IV) (Scheffler et al., 2014), however, it remains unclear whether the metabolic responses observed during market weight are parallel to muscle that is beginning to undergo robust hypertrophy.

To address this question, pigs carrying naturally occurring RyR1^{R615C} and AMPK γ ^{R200Q} mutations were bred to further characterize metabolic adaptations that occur earlier in the muscle growth curve. Defining these changes in metabolic regulation will provide insight into how nutrient utilization and mitochondrial function evolve throughout a pig's lifespan. This knowledge will aid in identifying key biochemical regulators that can be targeted to improve feed efficiency, enhance lean tissue accretion, and ultimately advance the sustainability and productivity of swine

production systems. Therefore, the objective of this study is to define the phenotypic and metabolic responses of skeletal muscle in pigs harboring mutations that drive oxidative and hypertrophic stimuli.

Materials and Method

Animal Harvest and Sample Collection

Pigs harboring the naturally occurring RyR1^{R615C} and AMPK γ ^{R200Q} mutations were bred to generate control (wild-type, WT), heterozygous RyR1^{R615C} (HAL), heterozygous AMPK γ ^{R200Q} (RN), and heterozygous AMPK γ ^{R200Q} + heterozygous RyR1^{R615C} (RN-HAL) littermates. These pigs were bred and reared under standard conditions and had ad libitum access to a corn-soybean meal-based diet at the Virginia Tech Swine Center, and all procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee. At 104 days of age (averaging ~47.6 kg), pigs were transported to the Virginia Tech Meat Science Center. Thirty-two pigs (8 pigs of each genotype) of equal gender (16 barrows, 16 gilts) were harvested and samples were collected immediately after exsanguination from the *longissimus dorsi* (LD, glycolytic muscle), *latissimus dorsi* (LAT, mixed muscle), *semitendinosus* (ST, mixed muscle) and *masseter* (MS, oxidative muscle). Samples were divided and snap frozen in liquid nitrogen, frozen for histology, or used to isolate mitochondria. Snap frozen samples were stored at -80 °C until further analysis. Muscle samples for histology were mounted into Avantor Tissue-Tek cryomolds (VWR International, Radnor, PA, USA) containing Tissue-Tek Optimal Cutting Media (Fisher Scientific, Hampton, NH, USA), placed in liquid nitrogen-cooled isopentane, and stored at -80 °C. Mitochondria were immediately isolated for metabolite tracing. After 24 hr, loin eye area and backfat thickness were measured.

Genotype Determination

Genotypes were verified ante- and postmortem using the PCR restriction fragment length polymorphism technique (Scheffler et al., 2014). Briefly, a portion of the pig tail (40 mg) was dissolved in lysis buffer containing 100 mM tris base (pH 8.5), 10 mM EDTA, 200 mM NaCl and 0.2% SDS and 2% proteinase K. Pig tail samples were incubated at 56 °C overnight followed by centrifugation at $13,000 \times g$ for 5 minutes. DNA was isolated from tail or muscle tissue (100 mg powdered tissue) by the Quick-DNA Miniprep kit (Zymo Research, Tustin, CA, USA) according to the manufacturer's instructions. Isolated DNA was used for PCR amplification. Each sample was amplified with RN and Hal primers:

Oligonucleotide	Sequence (5' to 3')
RN forward primer	AAATGTGCAGACAAGGATCTC
RN reverse primer	CCCACGAAGCTCTGCTT
Hal forward primer	TTCACCGGAGTGGAGTCTCTGAGT
Hal reverse primer	TCCAGTTTGCCACAGGTCCTACCA

PCR products with RN primers were digested with the BsrBI restriction enzyme (Cat # R0539S, New England Biolabs, Ipswich, MA, USA) for 1 hr at 37 °C and heat inactivated at 80 °C for 20 min. PCR products with Hal primers were digested with the HinPII restriction enzyme (Cat # R0124S, New England Biolabs, Ipswich, MA, USA) for 1 hr at 37 °C and heat inactivated at 65 °C for 20 min. DNA fragments were separated on a 1% agarose gel stained with SYBR safe DNA gel stain (ThermoFisher Scientific, Waltham, MA, USA) for visualization and imaged with the IBright Imaging System (ThermoFisher Scientific, Waltham, MA).

Mitochondrial Isolation

Mitochondria from the LD and MS muscles were isolated using the differentiation and derivative protocol described by (Scheffler et al., 2014; Matarneh et al., 2021; Rimmer et al., 2024). Fresh muscle (~40 g) was placed in ice-cold homogenization buffer (pH 7.4) containing 100mM sucrose, 180mM KCl, 50 mM tris-base, 5 mM MgCl₂, 1 mM K-ATP and 10 mM of 0.5M EDTA. Muscle was weighed and finely minced with scissors at a 1:3 (wt/vol) in homogenization buffer. Minced tissue was homogenized using a Potter-Elvehjem tissue homogenizer system (Glass-Col, Terre Haute, IN, USA), diluted with additional 20 mL of homogenization buffer and filtered through double-layered cheesecloth. The filtered solution was centrifuged at 4,000 × g for 10 min at 4 °C. Samples were filtered through cheesecloth once more and centrifuged at 10,000 × g for 10 min at 4 °C. Following centrifugation, the supernatant was discarded and the mitochondrial pellets were resuspended in a modified KHEB buffer (pH 7.2) containing 120 mM KCl, 0.2 mM KH₂PO₄, 1 mM HEPES and 0.5 mM EGTA. Total protein quantification of the isolated mitochondria was assessed using the Thermo Scientific™ Pierce™ BCA Protein Assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

Mitochondrial Respiration

Mitochondria from the LD and MS muscles were isolated through differential centrifugation as described above with a minor change. After mincing, muscle tissue was incubated with protease from *Bacillus licheniformis* (Cat #. 9014-01-1, Sigma Aldrich, St. Louis, MO, USA) for 10 min prior to homogenization. Mitochondrial protein concentration was determined by Thermo Scientific™ Pierce™ BCA Protein Assay Kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Isolated mitochondria were diluted to 1 µg/uL with mannitol sucrose medium (220 mM mannitol, 70 mM sucrose, 10 mM Tris-HCl, and 1 mM EGTA, pH 7.4).

Mitochondrial respiration was determined using Seahorse XFe96 (Agilent, Santa Clara, CA, USA) as described by (Yen et al., 2024). Respiration rate was assessed using at the following concentrations per well using multiple substrates: 1.5 μ g LD and 1 μ g MS were mixed with 10 mM pyruvate and 5 mM malate (PM), 1 μ g LD and MS were mixed with 10 mM succinate and 2 μ M rotenone (SR), and 2 μ g LD and MS were mixed 10 mM glutamate and 5 mM malate (GM). Mitochondrial oxygen consumption rate (OCR) was measured in a series of injections to target electron transport chain function. Baseline represents basal respiration of isolated mitochondria with incubated substrates. To assess oxidative phosphorylation capacity 4 mM ADP (Sigma-Aldrich, Darmstadt, Germany) was added to determine ADP-stimulated respiration. This step added ADP to promote ATP production through the ATP synthase complex (complex V). Proton leak was determined with 2 μ M oligomycin (Tocris Bioscience, Bristol, UK) since oligomycin inhibits complex V. The uncoupler carbonyl cyanide 4 (trifluoromethoxy) phenylhydrazone (FCCP; Sigma-Aldrich, Darmstadt, Germany) was added to a final concentration of 4 μ M to measure for maximal respiration. Non-mitochondrial respiration was determined with the 4 μ M antimycin A (Sigma-Aldrich, Darmstadt, Germany).

In vitro [¹³C₃]-Pyruvate Tracing in Enriched Mitochondria

To investigate pyruvate utilization in isolated mitochondria, an *in vitro* pyruvate tracing model was conducted as described by (Rimmer et al., 2024). Briefly, 3 mg of isolated mitochondria were added to 1 mL of KHEB containing 1 mM malic acid, 5 mM ADP, and 1 mM sodium pyruvate-¹³C₃ (Sigma Aldrich, St. Louis, MO, USA) in a 2 mL microcentrifuge tube. Unlabeled control reactions contained 1 mM sodium pyruvate. Mitochondria were incubated at 37 °C on an orbital rocker (New Brunswick Excella E24 Incubator Shaker, Marshall Scientific, Hampton,, NH, USA) at 40 rpm for 45 min. The incubated samples were mixed with 2 M perchloric acid, incubated

on ice for 15 min, and centrifuged at $12,000 \times g$ for 5 min. The supernatant was transferred to new tubes containing 3 M KOH to neutralize the reaction, and stored at $-80\text{ }^{\circ}\text{C}$ until further analysis.

Mass Spectrometry of TCA intermediates

Isolated mitochondria incubated with labeled pyruvate and tricarboxylic acid (TCA) intermediate standards were prepared for gas chromatography–mass spectrometry (GM-MS) using the deproteinization and derivatization method described in (Matarneh et al., 2021; Rimmer et al., 2024). Briefly, 5 M hydroxylamine hydrochloride was added to the samples followed by the addition of 4 M KOH. At a basic pH, the samples were sonicated for 1 hr at $60\text{ }^{\circ}\text{C}$, cooled, and then acidified with 6 N HCl. Ethyl acetate was added to samples to extract the organic phase of the supernatant after centrifugation. The samples were evaporated to dryness under compressed N_2 at $40\text{ }^{\circ}\text{C}$. Once dried, equal amounts of N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) and ethyl acetate were added to form the tertiary-butyldimethylsilyl (tBDMS) derivatives during a 1 h incubation period at $65\text{ }^{\circ}\text{C}$. The samples were separated using gas chromatography (Agilent 6890N; Agilent, Santa Clara, CA, USA) on a J&W HP-5 ms GC Column (HP-5MS, 30 m, 0.25 mm, 0.25 μm film; Agilent Technologies), as previously described in (Matarneh et al., 2021; Rimmer et al., 2024) with minor modifications. Intermediate ions were evaluated based on their mass-to-charge ratio (m/z) using the single ion monitoring method with a mass spectrophotometer (Agilent 5973N; Agilent, Santa Clara, CA, USA). The ions monitored included the following: lactate (261.10), pyruvate (274.10); succinate (289.10); fumarate (287.10); malate (419.20); oxaloacetate (432.10); α -ketoglutarate (446.20); and citrate (591.40). The mole percent excess (MPE) was determined through the peak area and was calculated as $(M + i/M + 0) \times 100$, where $M + i$ was defined as the isotopic enrichment of a molecule.

Western Blotting

Muscle samples were powdered in liquid nitrogen, homogenized in ice-cold IntactProtein™ lysis buffer (GenuIN Biotech, Blacksburg, VA, USA) according to manufacturer's instructions, and centrifuged at $13,000 \times g$ for 10 min at 4 °C. The supernatants were collected and protein concentration was determined with a BCA Protein Assay Kit, as mentioned previously. The samples were diluted to 3 mg/mL in 2X Laemmli sample buffer containing 2-mercaptoethanol, sodium dodecyl sulfate, glycerol, tris-hydrochloride, 0.05% bromophenol blue, protease inhibitor mini tablets (Cat #. PIA32955, Fisher Scientific, Hampton, NH, USA), and phosphatase inhibitor mini tablets (Cat #. A32957, Fisher Scientific, Hampton, NH, USA). Equal amounts of protein (30 ug) were separated using SDS-PAGE and transferred to PVDF or nitrocellulose membranes. The membranes were blocked with OneBlock blocking buffer (Prometheus Protein Biology Products, Genesee Scientific, San Diego, CA, USA) and incubated with the corresponding primary antibody overnight at 4 °C. The secondary antibodies used included Goat anti-Rabbit IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ Plus 647, Invitrogen™ (Cat# PIA32733, Fisher Scientific, Hampton, NH, USA), and goat anti-rabbit IgG antibody (H + L) HRP conjugate (Cat# AP307P, Sigma Aldrich, St. Louis, MO, USA). The enzymes used for the Western blot analysis were as follows (primary antibody, primary antibody concentration, secondary antibody, secondary antibody concentration, blocking time and temperature, and image detection): lactate dehydrogenase (anti-LDH α (Cat #: NBPI-48336SS, Novus Biologicals, Centennial, CO, USA), 1:1,000, goat anti-rabbit Alexa Fluor™ Plus 647, 1:10,000, 30 min at room temperature, fluorescence); pyruvate dehydrogenase alpha 1 (anti-PDH α -1 (Cat # AV48137, Sigma Aldrich, St. Louis, MO, USA), 1:900, goat anti-rabbit HRP, 1:9,000, 2 h at room temperature, chemiluminescence); and citrate synthase (anti-CS (Cat #

TA308265, OriGene, Rockville, MD, USA), 1:900, goat anti-rabbit HRP, 1:9,000, 2 h at room temperature, chemiluminescence). Prior to imaging, ProSignal Pico ECL reagent (Prometheus Protein Biology, Genesee Scientific, San Diego, CA, USA) was added to the PVDF membranes and incubated for 45 sec at room temperature on a benchtop rocker. Membranes were imaged using the fluorescence or chemiluminescence setting with the IBright Imaging System (ThermoFisher Scientific, Waltham, MA, USA). Total protein abundance was measured with the Invitrogen™ No-Stain™ Protein labeling reagent (ThermoFisher Scientific, Walham, MA, USA) following the manufacturer's instructions and imaged using the universal setting on the IBright Imaging System. All images were analyzed using Thermo Fisher iBright Analysis Software and normalized to the total protein abundance.

To evaluate the protein abundance of pyruvate carboxylase, 100 mg of tissue was homogenized in cold titin buffer containing 8 M urea, 2 M thiourea, 3.5 M glycerol, 3% SDS (wt/vol), and 75 mM dithiothreitol using a bead homogenizer (BeadBlaster™ Homogenzier, Millipore Sigma, St. Louis, MO, USA). Samples were transferred to a clean microcentrifuge tube and centrifuged at $10,000 \times g$ for 5 min. After centrifugation, supernatants were collected, transferred to a clean microcentrifuge tube and heated at 60 °C for 10 min. Protein concentration was determined using the Bio Rad RC DC™ (reducing agent and detergent compatible) Protein Assay kit according to the manufacturer's instructions. Protein samples were diluted to 3 mg/mL with titin buffer containing 0.05% bromophenol blue, heated 60 °C for 10 min, and separated using SDS-PAGE for 45 min. Gels were transferred to PVDF membranes, blocked for 45 min and incubated with 1:800 pyruvate carboxylase (anti-PC (Cat# NBPI-49536SS, Novus Biologicals, Centennial, CO, USA) overnight on a rocker at 4 °C. After the primary antibody was rinsed, membranes were incubated at a 1:9,000 dilution with goat anti-rabbit HRP for 1 h at room

temperature. Membranes were incubated with ProSignal Pico ECL reagent, imaged and analyzed as described above.

Total Free Calcium

Extractable total free calcium concentration was determined using a Mettler Toledo ion calcium selective electrode (VWR, Radnor, PA, USA) connected to a Mettler Toledo Seven Compact dual pH and ion meter (Columbus, OH, USA) according to the methods described by (Hopkins and Thompson, 2001; Hwang et al., 2004; Dang et al., 2020). Three grams of frozen muscle sample (-80 °C) was transferred to a -20 °C freezer for two weeks prior to measurement. Samples were removed from freezer, placed on a 1 °C chilled glass plate, and finely sliced (~ 1-2 mm thick) using a cold razor blade. Sliced samples were centrifuged at $120,000 \times g$ for 35 min at 4 °C and 300 μ L of supernatant was transferred to a tube containing 6 μ L of 4 M KCl. Samples were warmed to 20 °C for 2 min to ensure temperature consistency across all samples, and then calcium levels were read in duplicate with a measurement range of 1M to 5×10^{-6} M.

Prior to reading calcium levels, a standard calibration curve was measured. To calibrate the electrode for the standard curve and samples, the probe was placed in 100 mg/L Calcium ISE standard solution (Cat # 97054-698, Avantor Sciences, Radnor, PA, USA) for 1 hr. To begin measuring the standard curve, 2 mL of 4 M KCl was added to 100 mL ultrapure water. Increasing volumes of 1mM calcium ISE standard solution were added to the base solution and measured in millivolts. Effective calcium concentrations were calculated from the millivolt readings, converted to log μ M of calcium and used to create a standard curve. Total free calcium for the samples was determined using the standard curve.

Histology

Five-micron thick sections were cut using a 550 HM micron (ThermoFisher Scientific, Waltham, MA) and placed on protonated microscope slides to evaluate muscle cross-sectional area. Slides were submerged in 5% hematoxylin (Fisher Scientific, Hampton, NH) for 5 min and rinsed in running deionized water for 30 sec, and then were submerged in 0.03% eosin (Fisher Scientific, Hampton, NH) for 1 min and rinsed with deionized water. Rinsed slides were submerged in increasing concentrations of ethanol (50%, 70%, 95% and 100%), dipped in xylene for 7 sec, dried with a Kimwipe (Kimberly-Clark Professional Kimtech Science, Fisher Scientific, Hampton, NH) and mounted with Eprelia™ Shandon-Mount (Fisher Scientific, Hampton, NH). At a 10x magnification, five images were taken per sample with a total of 100 muscle fibers quantified per muscle using a Nikon Eclipse Ti2 inverted microscope (Nikon, Melville, NY) and analyzed with Nikon-Elements software. Cross-sectional area (μm^2) from the five images were averaged to obtain a single value per sample for statistical analysis.

Metabolite Analysis

Glycogen, glucose, glucose-6-phosphate (G6P), and lactate concentrations were quantified using 96-well plate enzymatic analytical assays as previously described (Hammelman et al., 2003; Kirkpatrick et al., 2023). For glucose, G6P, and lactate analyses, approximately 100 mg of powdered muscle tissue was homogenized in 0.5 M perchloric acid using a bead homogenizer (BeadBlaster™ Homogenizer, Millipore Sigma, St. Louis, MO, USA). Homogenates were incubated on ice for 15 min to ensure complete lysis and subsequently centrifuged ($13,000 \times g$, 4 °C, 10 min). The resulting supernatant was transferred to a fresh microcentrifuge tube and neutralized with 2 M KOH. Glycogen samples were prepared using a modified protocol. Briefly,

powdered tissue was homogenized in 1.25 M hydrochloric acid, incubated at 90 °C for 2 h, centrifuged, and neutralized with 1.25 M KOH.

Glucose and G6P concentrations were determined by incubating samples in a reaction buffer containing 6.25% triethanolamine, 12.5% of 200 mM EDTA (pH 7.4), 3.5% of 1 M MgCl₂, and NADP (95% of total assay volume). Initial absorbance (OD₁) was measured at 350 nm. Samples were then incubated with 1.9 U glucose-6-phosphate dehydrogenase (G6PDH; Cat #10165875001, Sigma Aldrich, St. Louis, MO, USA) for 20 min prior to a second absorbance measurement (OD₂). Following this, samples were incubated for an additional 20 min with 0.95 U hexokinase (Cat #11426362001, Sigma Aldrich) and 11 mg/mL Na-ATP, and a final absorbance (OD₃) was recorded. Glucose-6-phosphate concentrations were calculated as OD₂ – OD₁, while glucose concentrations were calculated as OD₃ – OD₁, with both determined using standard curves.

Lactate concentrations were measured by diluting samples 1:4 in a reaction buffer containing 0.125 M 5 M HCl, 473 mM glycine, 2.92 mM β-nicotinamide adenine dinucleotide sodium salt, and 2.73% hydrazine. Samples were plated in triplicate, and initial absorbance (OD₁) was measured at 340 nm. Each sample was then incubated with 1.75 U lactate dehydrogenase (Cat #L2625, Sigma Aldrich) for 90 min at room temperature prior to recording the final absorbance (OD₂). Lactate concentrations were calculated as OD₂ – OD₁ using a standard curve.

Muscle glycogen concentration was determined by diluting samples 1:4 and incubating them in a reaction buffer containing 1.35% triethanolamine, 12.5% of 200 mM EDTA (pH 7.4), 3.5% of 1 M MgCl₂, and NADP (95% of total assay volume). Initial absorbance (OD₁) was measured at 350 nm. Samples were subsequently incubated with 35 μL G6PDH (1.9 U), 35 μL hexokinase (0.95 U), and 70 μL Na-ATP (11 mg/mL) for 20 min at room temperature. Triplicate aliquots were then transferred to a 96-well plate, and absorbance (OD₂) was measured at 350 nm.

Glycogen concentrations were calculated as $OD_2 - OD_1$ using a standard curve. All metabolite concentrations are expressed as $\mu\text{mol/g}$ of tissue.

Phosphofructokinase Activity

Phosphofructokinase activity was quantified using an established 96-well plate enzymatic assay described by (Feliu et al., 1976; England et al., 2014). Frozen powdered muscle samples (~100 mg) were homogenized in 1 mL of 100 mM potassium phosphate buffer (K_2HPO_4 , pH 7.4). Homogenates were maintained on ice for 15 min to facilitate extraction and then centrifuged at $13,000 \times g$ for 10 min at 4 °C. A 40 μL aliquot of the supernatant was added to 1.8 mL of reaction buffer containing 120 mM MES, 3.2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 mM K-ATP, 1 mM NADH, and 3 mM fructose-6-phosphate (Cat #F3627, Sigma Aldrich, St. Louis, MO, USA). To initiate the enzymatic reaction, an enzyme mixture composed of glycerol-3-phosphate dehydrogenase (1.2 U; Cat #10127752001, Sigma Aldrich), triosephosphate isomerase (0.12 U; Cat #T2391, Sigma Aldrich), and aldolase (1 U; Cat #A2714, Sigma Aldrich) were added to samples. Reaction slope was monitored in triplicate using a 96-well microplate reader, with absorbance recorded at 340 nm at 1 min intervals over an 11 min period. Enzyme activity was derived from the linear rate of PEP disappearance, and slopes of each reaction were obtained when R-squared (RSQ) values were greater than 0.99.

Statistical Analysis

The data were analyzed as a split-plot with a whole plot factorial design using a mixed-effects model with fixed effects of genotype, gender, muscle and their interaction, and a random effect of pig. Data were considered significant when $p \leq 0.05$ and considered a trend when $p \leq 0.10$. Means were separated by pairwise comparisons. Main effect means (genotype, gender, or muscle) are reported when the interactions were not significant. Western blot data were log

transformed to normalize the residuals. Data are presented as the back-transformed values. Data are presented as the least squares means $\pm$ standard error. All data were analyzed using R (version 4.2.1, Indianapolis, IN, USA).

Results

A genetic model was employed to determine the phenotypic and metabolic responses of skeletal muscle in pigs harboring mutations that drive oxidative and hypertrophic stimuli. Pigs with naturally occurring RyR1^{R615C} and AMPK γ ^{R200Q} mutations were bred to generate control (wild-type, WT), heterozygous RyR1^{R615C} (HAL), heterozygous AMPK γ ^{R200Q} (RN), and heterozygous AMPK γ ^{R200Q} + heterozygous RyR1^{R615C} (RN-HAL) littermates. Skeletal muscles were harvested at 104 d of age to investigate the metabolic response early in the skeletal muscle growth curve (Rimmer et al., 2024). Muscles were selected based on their inherent metabolic characteristics (LD, glycolytic; LAT, mixed; ST; mixed, MS, oxidative) to evaluate how metabolism varies across muscles representing distinct phenotypes.

Tissue Growth Characterization

To assess whether early postnatal muscle growth differed among pigs harboring mutations known to influence skeletal muscle metabolism, measurements of muscle and adipose tissue were collected prior to euthanasia. Loineye area (LEA) was greater in WT and HAL barrows compared to RN and RN-HAL barrows (Figure 3.1A; $p < 0.05$). Additionally, LEA increased in WT and HAL barrows relative to WT and HAL gilts (Figure 3.1A; $p < 0.05$). Backfat thickness (BF) did not differ by gender (Figure 3.1B; $p = 0.710$) or genotype (Figure 3.1C; $p = 0.997$). Similarly, muscle cross-sectional area (CSA) was not influenced by gender (Figure 3.1D; $p = 0.996$) or genotype (Figure 3.1E; $p = 0.672$). However, CSA differed among muscles, with the ST exhibiting the greatest CSA, the MS with the lowest, and the LD and LAT displaying intermediate values

(Figure 3.1F; $p < 0.05$). To assess whether increased intracellular calcium, a hallmark characteristic of the HAL phenotype, was detectable at this developmental stage, total free calcium was quantified. Free calcium did not differ by gender (Figure 3.1G; $p = 0.820$) or genotype (Figure 3.1H; $p = 0.263$). Differences were observed among muscles, where free calcium was greatest in the MS, followed by the ST, LAT, and LD (Figure 3.1I; $p < 0.05$). Overall, these data demonstrate that while LEA differed by genotype and gender; whereas, CSA, and free calcium were not influenced by genotype or gender but varied across muscle.

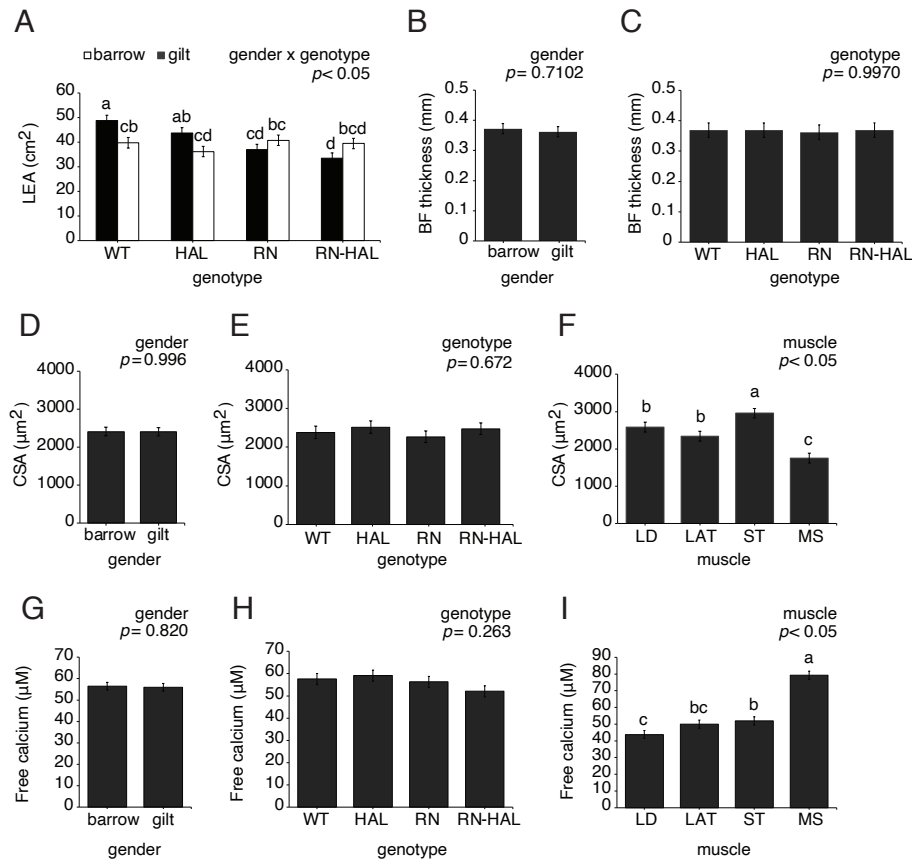


Figure 3.1. Growth characteristics of pigs harboring RyR1^{R615C} (HAL) and AMPK γ ^{R200Q} (RN) mutations. (A) Loineye area (LEA) of control (wild-type, WT), heterozygous RyR1^{R615C} (Hal), heterozygous AMPK γ ^{R200Q} (RN), and heterozygous AMPK γ ^{R200Q} + heterozygous RyR1^{R615C} (RN-

Hal) littermates. (B-C) Backfat thickness in each (B) gender and (C) genotype. (D-F) Cross-sectional area in each (D) gender, (E) genotype, and (F) muscle. (G-I) Total free calcium in each (G) gender, (H) genotype, and (I) muscle. Data are means $\pm$ SE. 8 pigs (4 barrows, 4 gilts) per genotype. Means lacking a common letter differ (a, b, c, d) when $p \leq 0.05$.

Assessment of Glycolytic Capacity and Glycolytic Enzymes in Skeletal Muscle

To assess glycolytic capacity, concentrations of glycogen, glucose, glucose-6-phosphate (G6P), and lactate were measured. Glycogen concentrations did not differ between gender (Figure 3.2A; $p = 0.441$) but increased in RN and RN-HAL pigs compared to WT and HAL pigs (Figure 3.2B; $p < 0.05$). Glycogen concentrations also differed among muscles, with the LD exhibiting the greatest levels, followed by the LAT, ST, and MS (Figure 3.2C; $p < 0.05$). Similarly, glucose concentrations were not influenced by gender (Figure 3.2D; $p = 0.697$). However, glucose levels were greatest in RN pigs compared to WT and HAL pigs, while RN-HAL pigs displayed intermediate values (Figure 3.2E; $p < 0.05$). Muscle differences were also observed, where glucose concentrations were highest in the LD, lowest in the MS, and intermediate in the LAT and ST (Figure 3.2F; $p < 0.05$). The concentrations levels of G6P did not differ by gender (Figure 3.2G; $p = 0.668$) but were increased in RN pigs compared to HAL pigs, with WT and RN-HAL pigs exhibiting intermediate concentrations (Figure 3.2H; $p < 0.05$). Consistent with glycogen and glucose patterns, G6P concentrations were greatest in the LD, lowest in the MS, and intermediate in the LAT and ST (Figure 3.2I; $p < 0.05$). Lactate concentrations were not influenced by gender (Figure 3.2J; $p = 0.407$) or genotype (Figure 3.2K; $p = 0.343$). However, differences were detected among muscles, where lactate concentrations were greatest in the LD, followed by the ST, LAT, and MS (Figure 3.2L; $p < 0.05$).

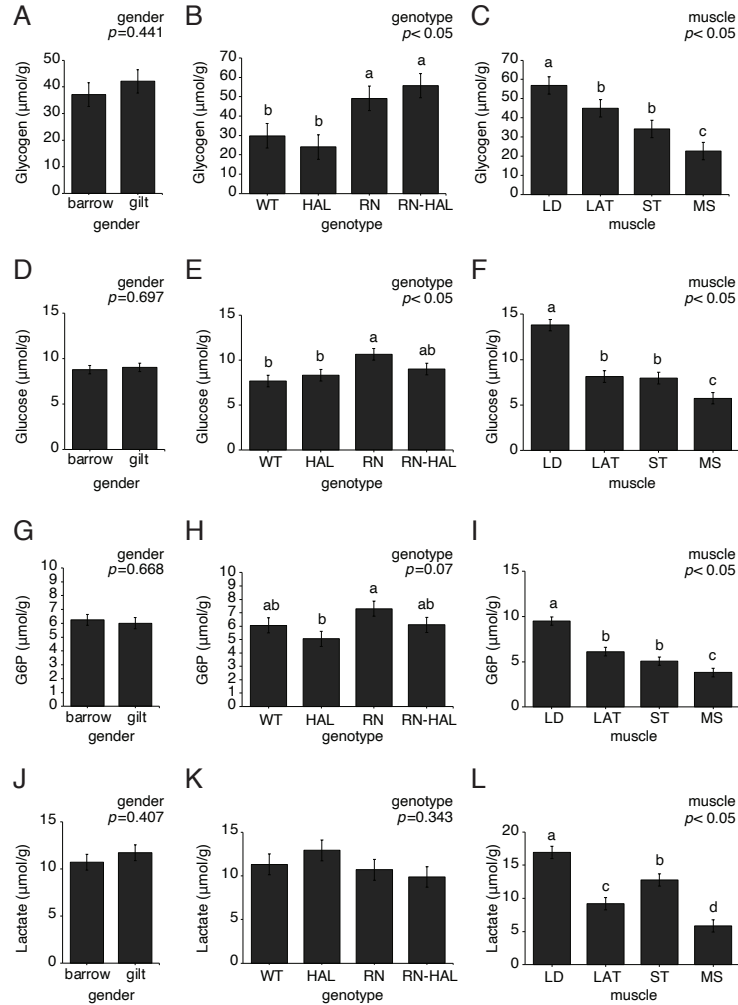


Figure 3.2. Glycolytic metabolite concentrations are altered by genotypes in pigs harboring RyR1^{R615C} (HAL) and AMPK γ ^{R200Q} (RN) mutations. (A-C) Glycogen concentrations in each (A) gender, (B) genotype, and (C) muscle. (D-F) Glucose concentrations in each (D) gender, (E) genotype, and (F) muscle. (G-I) Glucose-6-Phosphate (G6P) in each (G) gender, (H) genotype, and (I) muscle. (J-L) Lactate concentrations in each (J) gender, (K) genotype, and (L) muscle. Data are means $\pm$ SE. 8 pigs (4 barrows, 4 gilts) per genotype. Means lacking a common letter differ (a, b, c, d) when $p \leq 0.05$ and considered trending when $p \leq 0.10$.

The activity of phosphofructokinase (PFK) did not differ between gender (Figure 3.3A; $p = 0.403$); however, a significant muscle $\times$ genotype interaction was observed (Figure 3.3B; $p < 0.05$). PFK activity was greater in the LD muscles from WT and RN pigs compared to the LD muscles of HAL and RN-HAL pigs (Figure 3.3B; $p < 0.05$). Additionally, PFK activity was decreased in the LAT muscles of WT pigs compared to the LAT muscles of RN, HAL, and RN-HAL pigs (Figure 3.3B; $p < 0.05$). Conversely, PFK activity was increased in the ST muscles from RN and RN-HAL pigs compared to the ST muscles from WT pigs (Figure 3.3B; $p < 0.05$). These data indicate that PFK activity is not influenced by gender but is dependable on genotype and muscle. The abundance of lactate dehydrogenase alpha (LDH α) was highest in the LD, lowest in the MS and intermediate for LAT and ST muscle (Figure 3.3E; $p < 0.05$), although no differences were shown for gender (Figure 3.3C; $p = 0.153$) or genotype (Figure 3.3D; $p = 0.984$). The abundance of lactate dehydrogenase beta (LDH β) did not differ between genders (Figure 3.3F; $p = 0.388$); however, the abundance of LDH β in LD muscles from RN-HAL pigs increased compared to ST and MS muscles from RN-HAL pigs (Figure 3.3G; $p < 0.05$).

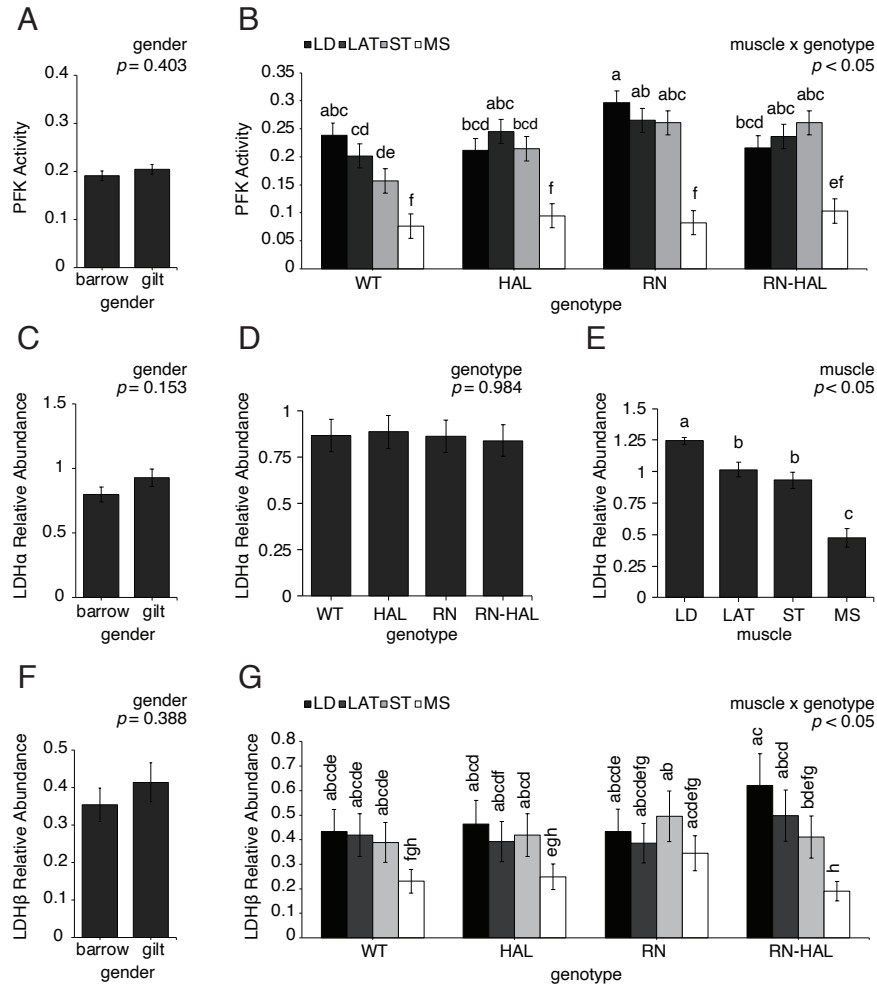


Figure 3.3. Assessment of glycolytic enzymes in pigs harboring RyR1^{R615C} (HAL) and AMPK γ ^{R200Q} (RN) mutations. (A-B) Phosphofructokinase (PFK) activity in each (A) gender and (B) genotype. (C-E) Relative abundance of lactate dehydrogenase alpha (LDH α) in each (C) gender, (D) genotype, and (E) muscle. (F-G) Relative abundance of lactate dehydrogenase beta (LDH β) in each (F) gender and (G) genotype. Data are means $\pm$ SE. 8 pigs (4 barrows, 4 gilts) per genotype. Means lacking a common letter differ (a, b, c, d) when $p \leq 0.05$.

Assessment of Mitochondrial Oxidative Enzymes

Pyruvate can also be transported into the mitochondrial matrix for oxidation within the TCA cycle. Upon entry, pyruvate has two primary metabolic fates: oxidation to acetyl-CoA via

pyruvate dehydrogenase (PDH) or carboxylation to oxaloacetate (OAA) via pyruvate carboxylase (PC). These pathways were evaluated due to their distinct implications for carbon retention. Specifically, PDH-mediated conversion results in CO₂ production, representing carbon loss, whereas PC-mediated carboxylation retains all carbon molecules. Additionally, the abundance of citrate synthase (CS), the first major enzyme of the TCA cycle that catalyzes the condensation of acetyl-CoA and OAA to form citrate, was assessed. The abundance of PDH α -1 did not differ between gender (Figure 3.4A; $p = 0.686$) or genotype (Figure 3.4B; $p = 0.559$); however, PDH α -1 abundance was greatest in the MS compared with all other muscles (Figure 3.4C; $p < 0.05$). Similarly, PC abundance was not influenced by gender (Figure 3.4D; $p = 0.290$) or genotype (Figure 3.4E; $p = 0.560$). Muscle effects were observed, with PC abundance highest in the LAT, lowest in the LD and MS, and intermediate in the ST (Figure 3.4F; $p < 0.05$). The abundance of CS was greatest in the MS, lowest in the LD, and intermediate in the LAT and ST (Figure 3.4I; $p < 0.05$), while no effects of gender (Figure 3.4G; $p = 0.909$) or genotype (Figure 3.4H; $p = 0.848$) were detected. Collectively, these data indicate that oxidative enzyme abundance was not influenced by genotype or gender at 104 d but was dependent on muscle metabolic phenotype.

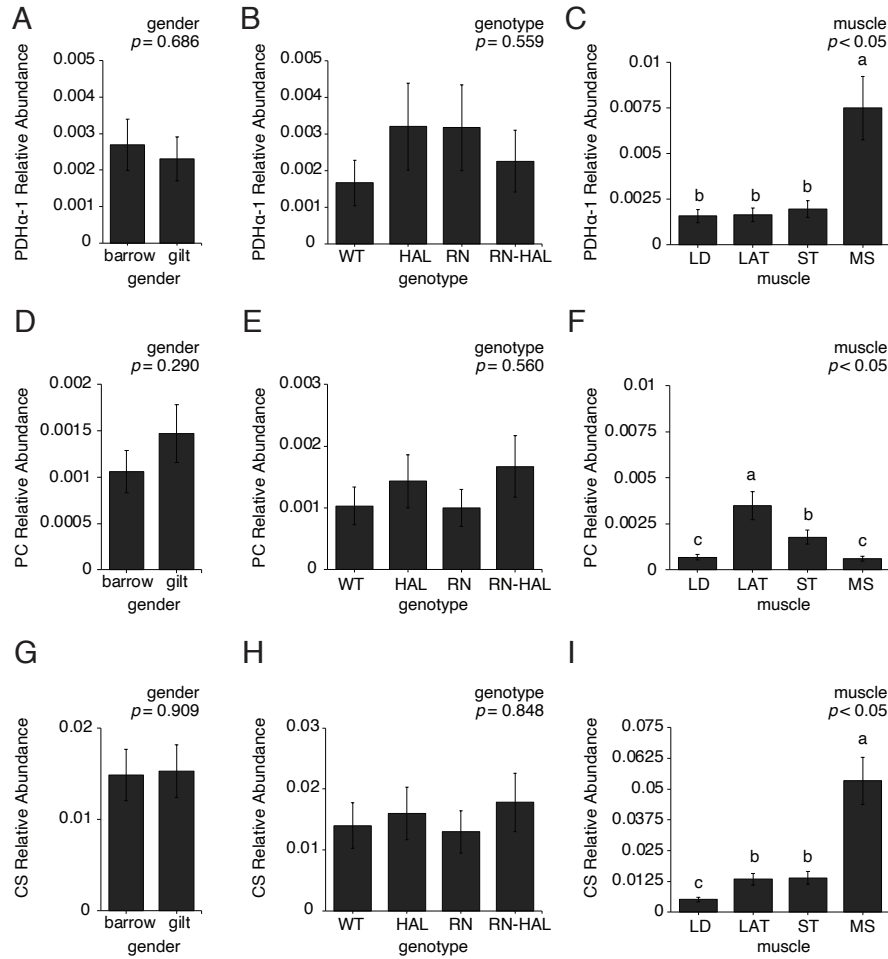


Figure 3.4. Assessment of key oxidative enzymes in the tricarboxylic acid (TCA) cycle in pigs harboring RyR1^{R615C} (HAL) and AMPK γ ^{R200Q} (RN) mutations. (A-C) Relative abundance of pyruvate dehydrogenase alpha-1 (PDH α -1) in each (A) gender, (B) genotype, and (C) muscle. (D-F) Relative abundance of pyruvate carboxylase (PC) in each (D) gender, (E) genotype, and (F) muscle. (G-I) Relative abundance of citrate synthase (CS) in each (G) gender, (H) genotype, and (I) muscle. Data are means $\pm$ SE. 8 pigs (4 barrows, 4 gilts) per genotype. Means lacking a common letter differ (a, b, c, d) when $p \leq 0.05$.

Assessment of Pyruvate-Derived TCA Intermediate Metabolism in the TCA Cycle

To further investigate how muscle distributes and utilizes pyruvate in pigs harboring HAL and RN mutations, we implemented an *in vitro* stable isotope tracing model adapted from (Matarneh et al., 2021; Rimmer et al., 2024). Mitochondrial enrichment preparations from LD and MS muscles were incubated with [$^{13}\text{C}_3$]-pyruvate, unlabeled malate, and ADP. Analysis of isotopomer enrichment patterns of TCA intermediates were analyzed (Figure 5) based upon output detected using GC-MS adapted from (Matarneh et al., 2021; Rimmer et al., 2024). Labeling patterns using a stable isotope tracer can be identified through subsequent TCA intermediate reactions (Inigo et al., 2021). Resulting M + i isotopomers reflect the incorporation of labeled carbons derived from pyruvate entering the TCA cycle through PDH (black circles) or PC (gray circles). For example, M + 3 citrate is indicative of three ^{13}C in the citrate molecule originating from the added labeled pyruvate (Figure 3). Enrichment fractions of lactate, pyruvate, citrate, alpha-ketoglutarate (AKG) and OAA derived from [$^{13}\text{C}_3$]-pyruvate are reported as mole percent excess.

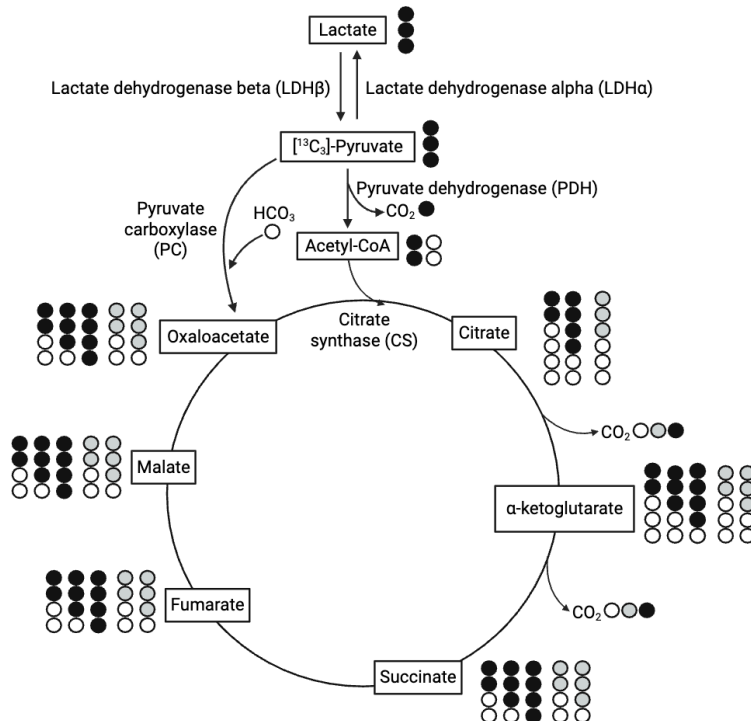


Figure 3.5. Schematic of potential labeling patterns of TCA intermediates after mitochondrial enrichment preparations from the LD, LAT, ST and MS were incubated with [$^{13}\text{C}_3$]-pyruvate, ADP, and malate. Black circles are ^{13}C derived from [$^{13}\text{C}_3$]-pyruvate that entered the TCA cycle through pyruvate dehydrogenase (PDH). Grey circles indicate ^{13}C derived from [$^{13}\text{C}_3$]-pyruvate that entered the TCA cycle through pyruvate carboxylase (PC). White circles represent unlabeled carbon atoms (^{12}C).

Lactate and Pyruvate Labeling Derived from [$^{13}\text{C}_3$]-Pyruvate

To evaluate carbon flux through the TCA cycle, isotopic enrichment of lactate M + 3 and pyruvate M + 3 was assessed. Lactate M + 3 did not differ between gender (Figure 3.6A; $p = 0.340$) or genotype (Figure 3.6B; $p = 0.981$) but was highest in the LD compared to the MS regardless of gender or genotype (Figure 3.6C; $p < 0.05$). No differences were observed for pyruvate M + 3 between gender (Figure 3.6D; $p = 0.805$), genotype (Figure 3.6E; $p = 0.759$), or muscle (Figure 3.6F; $p = 0.776$). These data indicate that pyruvate-derived carbon incorporation into lactate was muscle dependent, whereas pyruvate labeling remained consistent across gender, genotype, and muscle.

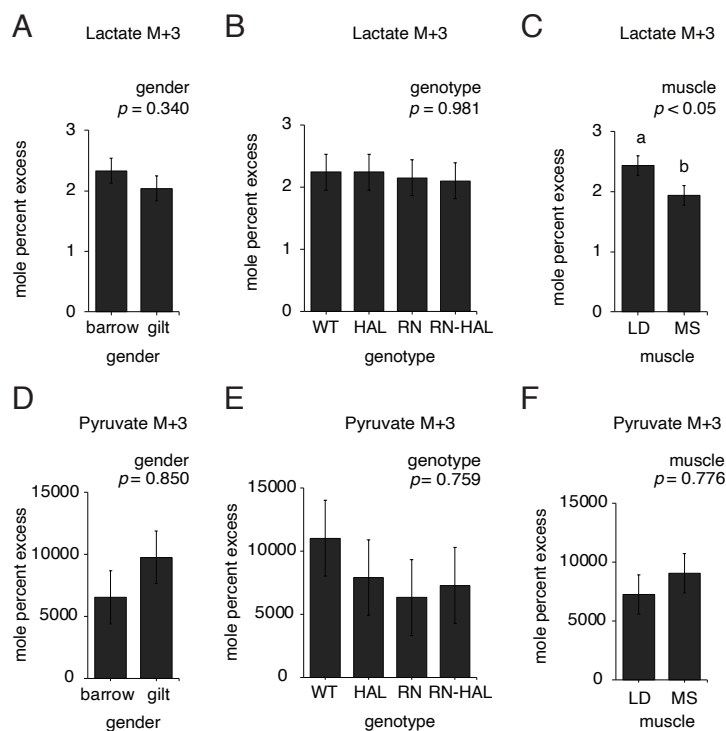


Figure 3.6. [$^{13}\text{C}_3$]-pyruvate derived isotopomer enrichment of lactate and pyruvate in mitochondrial enrichment preparations from pigs harboring RyR1^{R615C} (HAL) and AMPK γ ^{R200Q} (RN) mutations. (A-C) Enrichment of Lactate M + 3 in each (A) gender, (B) genotype, and (C) muscle. (D-F) Enrichment of Pyruvate M + 3 in each (D) gender, (E) genotype, and (F) muscle. Mitochondria were isolated from *longissimus dorsi* (LD) and *masseter* (MS) muscles of 4 barrows and 4 gilts per genotype (n=4). Means lacking a common letter differ (a, b, c, d) when $p \leq 0.05$.

Oxaloacetate Labeling Derived from [$^{13}\text{C}_3$]-Pyruvate

To further evaluate carbon flux through the TCA cycle, isotopic enrichment of OAA M + 2 and M + 3 was assessed. OAA M + 2 increased in barrow MS compared to gilt MS (Figure 3.7A; $p = 0.085$), whereas no differences were detected among genotypes (Figure 3.7B; $p = 0.564$). OAA M + 3 increased in gilt LD compared to barrow LD whereas barrow MS decreased compared to gilt MS (Figure 3.7C; $p = 0.093$). Additionally, OAA M + 3 was greater in RN and RN-HAL LD

compared to HAL LD (Figure 3.7D; $p = 0.098$). Collectively, these data suggest OAA labeling is dependent upon gender and muscle, with OAA M + 3 enrichment influenced by the RN genotype.

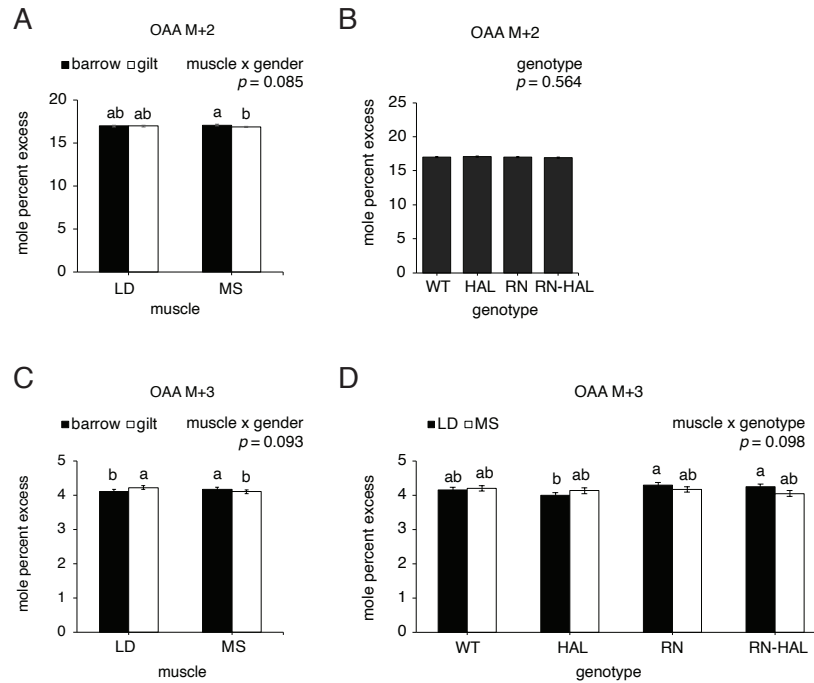


Figure 3.7. [$^{13}\text{C}_3$]-pyruvate derived isotopomer enrichment of oxaloacetate (OAA) in mitochondrial enrichment preparations from pigs harboring RyR1^{R615C} (HAL) and AMPK γ ^{R200Q} (RN) mutations. (A-B) Enrichment of OAA M + 2 in each (A) muscle and (B) genotype. (C-D) Enrichment of OAA M + 3 in each (C) muscle and (D) genotype. Mitochondria were isolated from *longissimus dorsi* (LD) and *masseter* (MS) muscles of 4 barrows and 4 gilts per genotype (n=4). Means lacking a common letter differ (a, b) when $p \leq 0.05$ and considered trending when $p \leq 0.10$.

Citrate Labeling Derived from [$^{13}\text{C}_3$]-Pyruvate

The next TCA intermediate evaluated for labeling patterns was citrate. Citrate M + 2 enrichment did not differ by gender (Figure 3.8A; $p = 0.541$) or genotype (Figure 3.8B; $p = 0.860$) but was greater in the MS compared to the LD (Figure 3.8C; $p < 0.05$). Similarly, citrate M + 3

was highest in the MS compared to the LD (Figure 3.8F; $p < 0.05$), while no differences were detected between gender (Figure 3.8D; $p = 0.539$) or genotype (Figure 3.8E; $p = 0.960$). These data suggest that citrate labeling is dependent upon muscle but not influenced by gender or genotype.

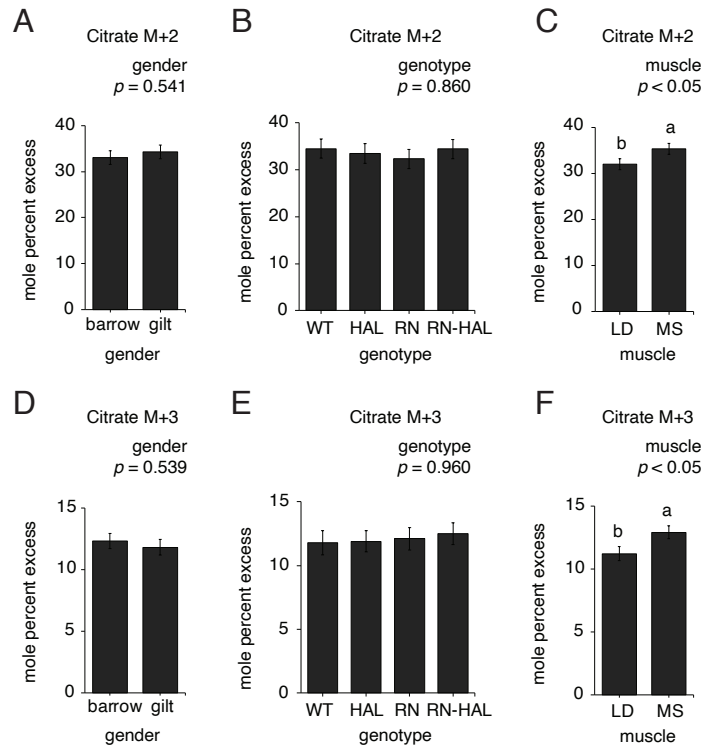


Figure 3.8. [$^{13}\text{C}_3$]-pyruvate derived isotopomer enrichment of oxaloacetate (OAA) in mitochondrial enrichment preparations from pigs harboring RyR1^{R615C} (HAL) and AMPK γ ^{R200Q} (RN) mutations. (A-C) Enrichment of citrate M + 2 in each (A) gender, (B) genotype, and (C) muscle. (D-F) Enrichment of citrate M + 3 in each (D) gender, (E) genotype, and (F) muscle. Baseline represents basal respiration of enriched mitochondria with substrates. Mitochondria were isolated from *longissimus dorsi* (LD) and *masseter* (MS) muscles of 4 barrows and 4 gilts per genotype (n=4). Means lacking a common letter differ (a, b) when $p \leq 0.05$.

Alpha-Ketoglutarate Labeling Derived from [¹³C₃]-Pyruvate

The final intermediate evaluated for labeling patterns was AKG. No differences were detected for AKG M + 2 (Figure 3.9A; $p = 0.899$) or AKG M + 4 (Figure 3.9C; $p = 0.375$). Enrichment of both AKG M + 2 and M + 4 was greatest in WT MS compared to WT LD (Figure 3.9B, D; $p = 0.06$). Together, these results reinforce that substrate flux through TCA intermediates is driven by muscle metabolic phenotype.

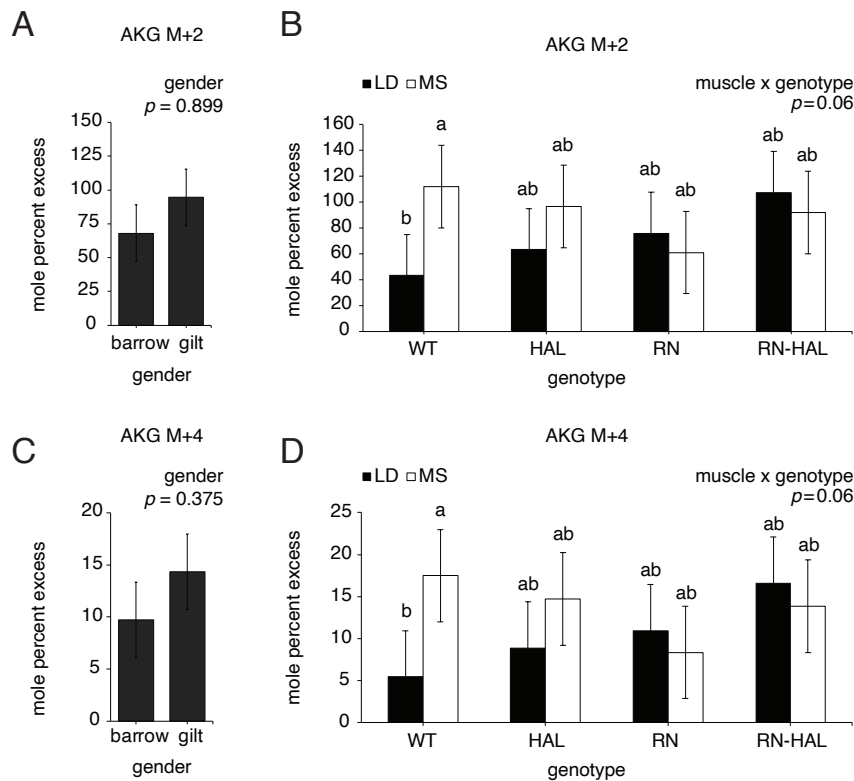


Figure 3.9. [¹³C₃]-pyruvate derived isotopomer enrichment of alpha-ketoglutarate (AKG) in mitochondrial enrichment preparations from pigs harboring RyR1^{R615C} (HAL) and AMPK γ ^{R200Q} (RN) mutations. (A-B) Enrichment of AKG M + 2 in each (A) gender and (B) genotype. (C-D) Enrichment of AKG M + 4 in each (C) gender and (D) genotype. Baseline represents basal respiration of enriched mitochondria with substrates. Mitochondria were isolated from *longissimus*

dorsi (LD) and *masseter* (MS) muscles of 4 barrows and 4 gilts per genotype (n=4). Means lacking a common letter differ (a, b, c, d) when $p \leq 0.05$ and considered a trend when $p \leq 0.10$.

Assessment of Oxygen Consumption Rate in Isolated Mitochondria

Mitochondria respiration (oxygen consumption rate; OCR) was measured in isolated LD and MS *in vitro* to further assess the impact that pigs harboring RyR1^{R615C} and AMPK γ ^{R200Q} mutations may have on mitochondrial functional oxidative capacity. Mitochondrial enrichment preparations were incubated with either pyruvate/malate (P/M) or succinate/rotenone (S/R) and stimulated with the substrates (ADP, FCCP, or oligomycin) to evaluate a comprehensive understanding of mitochondrial respiration. Basal P/M respiration did not differ between gender (Figure 3.10A; $p = 0.907$), but a muscle $\times$ genotype interaction was observed (Figure 3.10B; $p < 0.05$). Basal respiration from MS mitochondria from WT pigs increased compared to LD mitochondria from WT pigs (Figure 3.10B; $p < 0.05$) when provided with substrates pyruvate/malate. Conversely, LD mitochondria from RN-HAL pigs increased in basal P/M respiration compared to MS mitochondria from RN-HAL pigs (Figure 3.10B; $p < 0.05$). ADP stimulated P/M respiration did not differ by gender (Figure 3.10C; $p = 0.525$) or genotype (Figure 3.10D; $p = 0.644$); however, the MS mitochondria increased compared to the LD mitochondria (Figure 3.10E; $p < 0.05$). Similarly, FCCP stimulated P/M respiration increased in the MS mitochondria compared to the LD mitochondria (Figure 3.10H; $p < 0.05$), whereas no differences were detected between gender (Figure 3.10F; $p = 0.547$) or genotype (Figure 3.10G; $p = 0.507$). Oligomycin stimulated P/M respiration did not differ by gender (Figure 3.10I; $p = 0.547$) but resulted in a muscle $\times$ genotype interaction (Figure 3.10J; $p < 0.05$). Oligomycin stimulated P/M increased OCR in MS mitochondria from WT pigs compared to MS mitochondria from HAL and RN-HAL pigs (Figure 3.10J; $p < 0.05$). Basal S/R respiration did not differ in gender (Figure

3.11A; $p = 0.408$) or genotype (Figure 3.11A; $p = 0.536$). Mitochondrial respiration from the MS mitochondria increased compared to the LD mitochondria (Figure 3.11C; $p < 0.05$) when provided with substrates succinate/rotenone. ADP stimulated S/R respiration OCR increased in LD mitochondria from RN-HAL barrows compared to the LD mitochondria from RN-HAL barrows (Figure 3.11D; $p = 0.06$). Additionally, the LD mitochondria from HAL gilts and the MS mitochondria from RN gilts exhibited an increased OCR compared to the LD mitochondria from WT gilts (Figure 3.11D; $p = 0.06$). FCCP stimulated S/R OCR in MS mitochondria from RN and RN-HAL barrows increased compared to MS and LD mitochondria from WT barrows and LD mitochondria from RN barrows (Figure 3.11E; $p < 0.05$). In contrast FCCP stimulated S/R OCR in MS mitochondria from RN-HAL gilts decreased compared to LD mitochondria from WT gilts (Figure 3.11E; $p < 0.05$). Finally, oligomycin stimulated S/R respiration in LD mitochondria from HAL and RN gilts increased compared to LD mitochondria from WT gilts (Figure 3.11F; $p < 0.05$). Furthermore, MS mitochondria from HAL barrows increased compared to MS mitochondria from RN-HAL barrows (Figure 3.11F; $p < 0.05$). Collectively, these findings indicate that mitochondrial respiration is influenced by complex interactions among genotype, muscle, and gender.

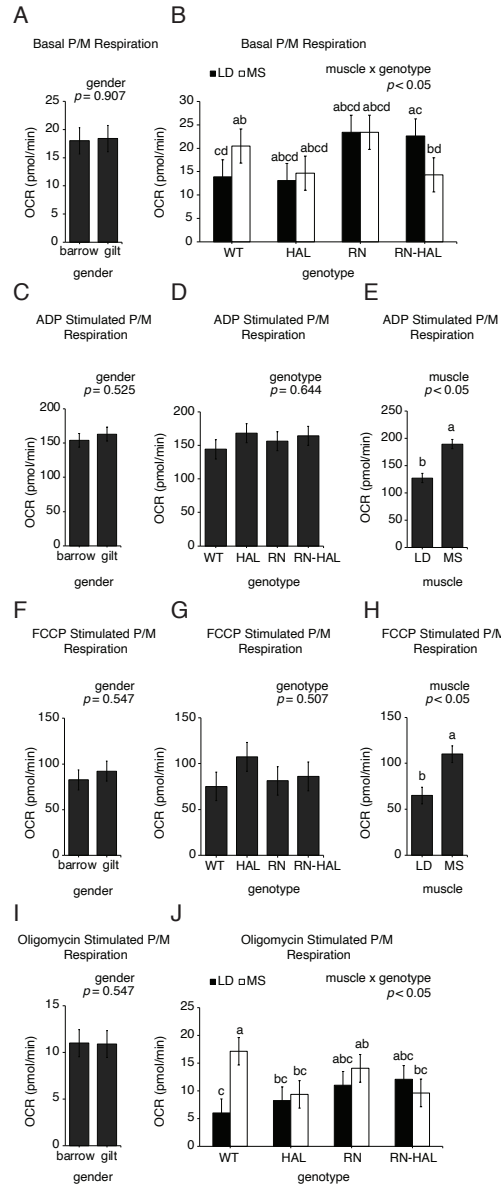


Figure 3.10. Oxygen consumption rate (OCR) of mitochondria isolated from *longissimus dorsi* (LD) and *masseter* (MS) muscles under saturating concentrations of pyruvate/malate (P/M) substrate from pigs harboring RyR1^{R615C} (HAL) and AMPK γ ^{R200Q} (RN) mutations. (A-B) Basal respiration in each (A) gender and (B) genotype. (C-E) OCR of mitochondria stimulated with adenosine diphosphate (ADP; 5 mM) in each (C) gender, (D) genotype, and (E) muscle. (F-H) OCR of mitochondria stimulated with carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone

(FCCP, 4 μ M) in each (F) gender, (G) genotype, and (H) muscle. (I-J) OCR of mitochondria stimulated with oligomycin (2 μ M) in each (I) gender and (J) genotype. Data are means $\pm$ SE. 8 pigs (4 barrows, 4 gilts) per genotype. Means lacking a common letter differ (a, b, c, d) when $p \leq 0.05$.

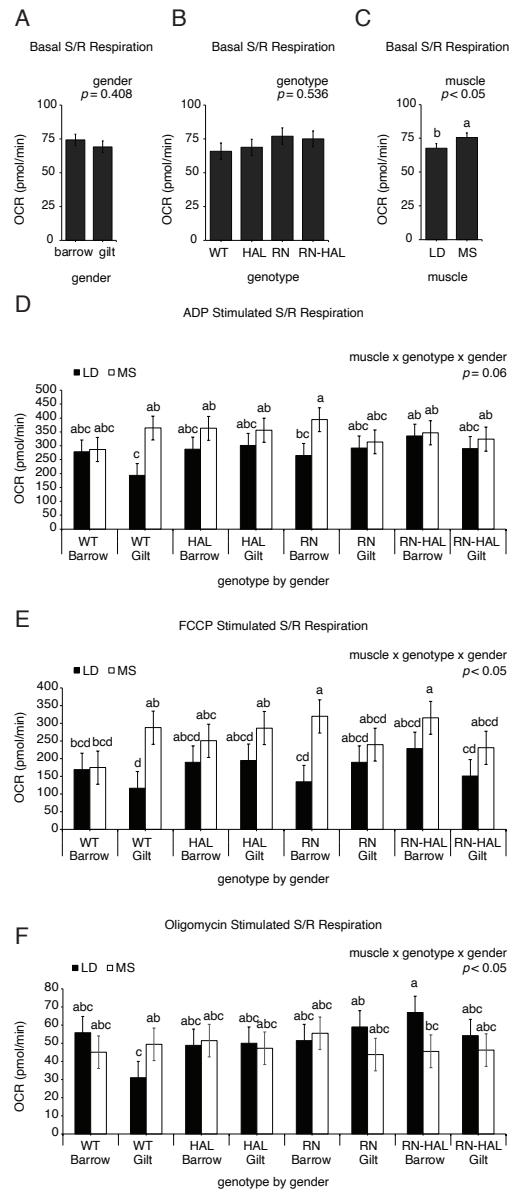


Figure 3.11. Oxygen consumption rate (OCR) of mitochondria isolated from *longissimus dorsi* (LD) and *masseter* (MS) muscles under saturating concentrations of succinate/rotenone (S/R)

substrate from pigs harboring RyR1^{R615C} (HAL) and AMPK γ ^{R200Q} (RN) mutations. (A-C) Basal respiration OCR in each (A) gender, (B) genotype and (C) muscle. (D) OCR of mitochondria stimulated with adenosine diphosphate (ADP; 5 mM). (E) OCR of mitochondria stimulated with carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, 4 μ M). (F) OCR of mitochondria stimulated with oligomycin (2 μ M). Data are means $\pm$ SE. 8 pigs (4 barrows, 4 gilts) per genotype. Means lacking a common letter differ (a, b, c, d) when $p \leq 0.05$ and considered trending when $p \leq 0.10$.

Discussion

Skeletal muscle metabolic profile determines muscle fiber types (Gerrard and Grant, 2003), which influence both energy utilization and growth potential (Gerrard and Grant, 2003; van Wessel et al., 2010). Oxidative fibers are characterized by a more vibrant red appearance, increased phospholipid content, smaller fiber size, and lower shear force compared to highly glycolytic fibers (Lee et al., 2016). Functionally, enhanced oxidative capacity supports sustained ATP production and protects against cellular energy deficits which ultimately improves muscle function (Chalkiadaki et al., 2014). However, oxidative capacity is inversely related to muscle fiber size (Gerrard and Grant, 2003; van Wessel et al., 2010), highlighting main distinctions between metabolic efficiency and hypertrophic potential. Interestingly, previous work has demonstrated that pigs harboring both the HAL and RN mutations (RN-HAL offspring) exhibit increased muscle hypertrophy at market weight while maintaining a more oxidative phenotype (Scheffler et al., 2014). While this study identified distinct metabolic differences among genotypes at market weight, the timing of when these metabolic shifts emerge during the onset of intense hypertrophy during postnatal muscle growth remains unclear. Rimmer et al. (2024) established the onset of muscle hypertrophy begins around 87 to 120 days of age and further revealed dynamic, age-

dependent metabolic changes throughout a pig's life. Therefore, the objective of this study was to determine how naturally occurring mutations in RyR1^{R615C} and AMPK γ ^{R200Q} genes influence skeletal muscle growth and metabolism during a developmental stage predicted to coincide with the onset of rapid hypertrophy, building upon findings from these previous studies (Scheffler et al., 2014; Rimmer et al., 2024).

To assess whether growth characteristics associated with HAL and RN mutations at market weight were evident at this earlier developmental stage, LEA, BF, and CSA were evaluated. Pigs harboring the HAL mutation have been shown to exhibit greater muscle hypertrophy at market weight compared to control pigs (Zhang et al., 1992), and RN-HAL pigs demonstrate increased hypertrophic potential relative to RN pigs (Scheffler et al., 2014). However, in the present study, muscle CSA did not differ among genotypes ($p = 0.672$), likely reflecting an early harvest timepoint prior to the onset of pronounced muscle hypertrophy (Rimmer et al., 2024). These data suggest that these naturally occurring mutations impact muscle phenotype during the intense hypertrophic developmental stage closer to market weight. Similarly, no differences were detected in BF thickness ($p = 0.997$), suggesting that pigs were harvested before substantial adipose accretion had occurred, consistent with previous reports describing the progression of adipose deposition during development (Rimmer et al., 2024). To further evaluate early physiological differences, total free calcium was measured to determine whether alterations in sarcoplasmic calcium handling were present, given that the HAL mutation is known to increase intracellular calcium concentrations (Kathirvel and Archibald, 2001). Although no genotype differences were observed ($p = 0.263$), free calcium concentrations were greater in the MS compared to all other muscles ($p < 0.05$). These findings suggest that oxidative muscles may be inherently more sensitive to subtle changes in calcium regulation, even prior to the full manifestation of genotype-specific

phenotypes (Murgia et al., 2021). Collectively, these results support that 104 d of age represents a transitional developmental stage in which metabolic differences are emerging, while morphological traits associated with muscle hypertrophy and adipose deposition have not yet fully developed.

To evaluate how these naturally occurring mutations influence glycolytic metabolism in skeletal muscle, glycolytic metabolites (glycogen, glucose, G6P and lactate) and enzymes PFK, LDH α , LDH β were measured. Glycogen serves as the primary carbohydrate reserve in skeletal muscle and can be converted to glucose through glycogenolysis to support glycolytic flux. Glucose entering glycolysis is phosphorylated to G6P, a central metabolic intermediate. The next major step within glycolysis is carried out by PFK, a key regulatory enzyme, to produce fructose 1,6-bisphosphate. This enzyme is often used as an indicator of glycolytic capacity (Tornheim and Lowenstein, 1976). Glycolysis culminates in the production of pyruvate through the phosphorylation of phosphoenolpyruvate by pyruvate kinase. Pyruvate can be reduced to lactate by LDH α , allowing lactate to be transported to the liver where it may undergo gluconeogenesis through the Cori cycle. The resulting glucose can subsequently reenter glycolytic pathways. Conversely, lactate can be converted back to pyruvate by LDH β . In fact, LDH β is commonly evaluated as an indicator of oxidative capacity and metabolic flexibility, reflecting the ability of muscle fibers to shift between glycolytic and oxidative metabolism (Elustondo et al., 2013). Collectively, these metabolites and enzymes provide an assessment of glycolytic and partial oxidative metabolic characteristics across muscles. Glycogen concentrations were highest in RN and RN-HAL pigs compared to WT and HAL pigs ($p < 0.05$), consistent with previous reports describing elevated glycogen storage in pigs harboring mutations in the RN gene (Scheffler et al., 2016). This response is expected, as the AMPK γ mutation is well-established to enhance glycogen

accumulation in skeletal muscle (Costford et al., 2007; Scheffler et al., 2016). Independent of genotype, glycogen content varied by muscle, with the highest levels observed in the LD, the lowest in the MS, and intermediate concentrations in the LAT and ST ($p < 0.05$). These findings align with established metabolic differences among muscles, particularly the greater glycogen storage capacity of muscles composed mostly of type II fibers (De Bock et al., 2007). Glucose concentrations were highest in RN pigs relative to WT and HAL pigs ($p < 0.05$), while RN and RN-HAL pigs exhibited similar glucose levels, suggesting the RN mutation contributes to elevated glucose levels. Similarly, G6P concentrations tended to increase in RN pigs compared with HAL pigs ($p = 0.07$). Interestingly, Scheffler et al. (2016) previously reported greater G6P concentrations in HAL pigs at market weight. The discrepancy between studies may indicate temporary differences in metabolic regulation, suggesting that the HAL mutation may alter glycolytic flux differently across developmental stages.

To further evaluate glycolytic regulation, PFK activity was assessed. The activity of PFK was reduced in the LD muscles from HAL and RN-HAL pigs compared to the LD muscles from RN pigs ($p < 0.05$), indicating that the HAL mutation modifies glycolytic control in a muscle-dependent manner. Given the central regulatory role of PFK, these data suggest a potential attenuation of glycolytic capacity in HAL-containing genotypes. One plausible interpretation is that metabolites may be redirected toward biosynthetic pathways branching from glycolysis, thereby supporting anabolic processes associated with muscle hypertrophy (Wagner et al., 1978; Battistuzzi et al., 1985; Zumbaugh et al., 2022). Despite these alterations in upstream glycolytic metabolites, lactate concentrations did not differ among genotypes ($p = 0.343$), consistent with the lack of genotype effects on LDH α abundance ($p = 0.984$). However, muscle-specific differences emerged, as the LAT muscles from RN and RN-HAL pigs exhibited greater lactate concentrations

relative to the MS muscles from RN and RN-HAL pigs ($p < 0.05$). This pattern suggests variation in glycolytic end-product routing that is influenced by both genotype and muscle metabolic phenotype. Collectively, these findings indicate that RN and HAL mutations exert both distinct and interacting effects on glucose metabolism. While the RN mutation primarily enhances substrate availability through increased glycogen and glucose concentrations, the HAL mutation appears to influence glycolytic regulation in a muscle-specific manner. These data collectively highlight early metabolic adaptations that may ultimately shape future muscle growth and functional characteristics.

Alternatively, pyruvate can be transported into the mitochondrial matrix, where it undergoes oxidation within the TCA cycle. Entry into the TCA cycle occurs through two primary routes, as described above. Previous studies have demonstrated that PC is upregulated while PDH is downregulated, in high feed-efficient pigs (Fu et al., 2017). Indeed, the HAL mutation has been associated with improved feed efficiency and enhanced growth characteristics (Silveira et al., 2011). Additionally, the RN mutation is known to increase oxidative enzymes (Scheffler et al., 2014; Scheffler et al., 2016), therefore, we evaluated key oxidative enzymes (PDH α 1, PC and CS) attributed to the TCA cycle. However, no genotype differences were detected in PDH α 1 ($p = 0.559$), PC ($p = 0.560$), or CS ($p = 0.848$), suggesting that nutrient flux into the TCA cycle is not altered at this stage of development. Although the RN genotype has been associated with increased oxidative enzyme capacity (Scheffler et al., 2014; Scheffler et al., 2016), the energetic demands during this early developmental period may not yet necessitate enhanced oxidative metabolism. Indeed, the energy required for rapid muscle accretion far exceeds that needed for maintenance, with approximately 5,000 kJ required to support the growth of 1 kg of muscle compared to ~55 kJ required to maintain 1 kg of existing muscle (Slater et al., 2019). Given that pigs in the present

study were harvested prior to the onset of robust muscle hypertrophy, it is likely that genotype-dependent differences in energy demand and oxidative metabolism had not yet fully manifested.

To further characterize pyruvate metabolism in skeletal muscle, an *in vitro* isotope tracing model was performed. Mitochondrial enrichment preparations from LD and MS muscles were incubated with [$^{13}\text{C}_3$]-pyruvate in the presence of ADP and malate, and isotopomers of TCA cycle intermediates were quantified by GC-MS to determine mole percent excess (Figure 5). Lactate M + 3 did not differ in gender ($p = 0.340$) or genotype ($p = 0.981$); however, enrichment was greater in LD muscles compared with MS muscles ($p < 0.05$). These data suggest that the LD muscle may preferentially direct pyruvate towards lactate formation rather than mitochondrial oxidation in order to preserve glycolytic substrates or to support biosynthetic processes (Mantyselka et al., 2025). To evaluate substrate utilization in skeletal muscle, pyruvate M + 3 was measured as an indicator of total pyruvate uptake. Pyruvate M + 3 did not differ among genotypes ($p = 0.759$) or muscle ($p = 0.776$), indicating that mitochondrial pyruvate uptake and utilization were not substantially altered at the timepoint evaluated. OAA M + 2 was greater in barrow MS muscles compared to gilt MS muscles ($p = 0.085$), while OAA M + 3 differed between gender in a muscle-specific manner ($p = 0.093$). In isotope tracing experiments with a fully labeled (M + 3) pyruvate entering the TCA cycle, an increase in M + 2 citrate suggests an increase in pyruvate entering the TCA cycle through PDH (Gravel et al., 2014; Duan et al., 2022; Khattri et al., 2022; Gopal et al., 2023). On the other hand, if pyruvate enters the TCA cycle via PC, pyruvate is carboxylated to oxaloacetate by consuming HCO_3^- , which produces M + 3 oxaloacetate (Figure 5, grey circles). Citrate M + 2 did not differ between gender ($p = 0.541$) or genotype ($p = 0.860$); however, the MS was greater than the LD ($p < 0.05$). Although genotype effects were not detected, the muscle effect is consistent with the inherently greater oxidative capacity of the MS. This observation aligns with

previous reports demonstrating elevated citrate M + 2 enrichment in MS relative to LD muscles in pigs of similar developmental age (Rimmer et al., 2024). These data align with our PDH α -1 results where the abundance was greater in the MS compared to the LD ($p < 0.05$). Similarly, citrate M + 3 did not differ in gender ($p = 0.539$) or genotype ($p = 0.960$), while the MS increased compared to the LD ($p < 0.05$). Interestingly, distinct genotype patterns emerged for AKG labeling. AKG M + 2 and M + 4 increased in MS muscles from WT pigs compared to LD muscles from WT pigs while no differences were discernable between LD and MS muscles in all other genotypes ($p = 0.06$). Together, these findings indicate that RN and HAL mutations may influence the partitioning of pyruvate-derived carbon within the TCA cycle, potentially modifying oxidative metabolism without altering total pyruvate consumption.

While isotope tracing provided insight into carbon partitioning (Inigo et al., 2021), mitochondrial respiration was evaluated to determine whether oxidative capacity differed among gender, genotype, and muscle. Given that swine are fed high carbohydrate-based diets (Navarro et al., 2019), a substantial proportion of nutrients are metabolized through glycolysis, generating pyruvate as a central metabolic intermediate (Xiang et al., 2021). Pyruvate and malate support respiration through Complex I and enable assessment of whole-chain ETC activity, whereas succinate in the presence of rotenone drives Complex II respiration, as rotenone inhibits Complex I (Boutagy et al., 2015). Baseline respiration was measured with pyruvate/malate or succinate/rotenone prior to the addition of substrates. ADP and FCCP stimulated P/M respiration did not differ by gender or genotype, whereas OCR was greater in the MS compared with the LD ($p < 0.05$). This pattern aligns with established metabolic distinctions between these muscles, as the inherently oxidative MS consistently exhibits greater respiratory capacity than the more glycolytic LD, independent of substrate conditions (Yen et al., 2024). Although previous work

demonstrated that RN pigs at market weight display elevated ADP stimulated P/M respiration relative to WT and HAL pigs (Scheffler et al., 2014), such differences were not evident at this developmental stage. Consistent with the results from the glycolytic metabolites, these findings suggest that the RN phenotype may employ a more pronounced influence later in growth rather than during early development. In contrast, oligomycin stimulated P/M respiration reduced OCR in HAL and RN-HAL MS compared with WT MS ($p < 0.05$). Because oligomycin inhibits ATP synthase, this reduction suggests tighter coupling of oxidative phosphorylation in HAL-containing pigs, potentially reflecting reduced proton leak and enhanced mitochondrial efficiency at this age (Leverve and Fontaine, 2001). Interestingly, distinct patterns resulting in gender $\times$ genotype $\times$ muscle interactions emerged under S/R stimulated respiration.

Scheffler et al. (2014) demonstrated that the RN mutation increases ADP stimulated respiration, regardless of substrate (P/M or S/R). In the present study, no genotype differences were observed in ADP-stimulated respiration using P/M ($p > 0.05$). However, a muscle-specific effect was detected in our ADP stimulated S/R respiration ($p = 0.06$) where OCR was increased in the MS muscle of RN barrows compared to the LD muscle of RN barrows and the LD muscle of WT gilts. These findings suggest that, at this developmental stage, mitochondrial respiratory capacity may be influenced more by muscle-specific metabolic demand than by genotype alone, potentially reflecting early, localized adaptations in oxidative metabolism. Additionally, previous reports identified that oligomycin markedly reduced oxygen consumption in RN pigs (Scheffler et al., 2014). Consistent with these findings, the present study also showed minimal differences in oligomycin stimulated respiration. However, LD muscle from RN-HAL barrows exhibited increased OCR compared to LD muscle from WT gilts ($p < 0.05$), suggesting a potential gender-dependent effect on mitochondrial respiration. The overall similarity in oligomycin respiration

across genotypes indicates that mitochondrial coupling efficiency is potentially preserved at this stage of growth. Collectively, these results suggest that while RN-associated increases in oxidative capacity may emerge later in development, early differences in mitochondrial respiration are subtle, muscle-specific, and not yet indicative of broad alterations in mitochondrial efficiency. Overall, these findings suggest that genotype-dependent differences in mitochondrial function are apparent during early development and are influenced by both muscle metabolic phenotype and gender under different substrate conditions. Together, these data reinforce that mitochondrial adaptations associated with the RN and HAL phenotypes may evolve with age, becoming more apparent as pigs reach market weight.

Conclusion

In summary, this study demonstrates that naturally occurring mutations in the RyR1^{R615C} (HAL) and AMPK γ ^{R200Q} (RN) genes contribute to metabolic adaptations in skeletal muscle prior to the emergence of overt phenotypic differences often associated with these genotypes. Although muscle morphology and adipose deposition were not altered at 104 d of age, distinct shifts were observed in glycolytic regulation and potential, pyruvate carbon partitioning, and mitochondrial bioenergetics. Importantly, mitochondrial functional differences were detectable despite minimal changes in protein abundance of key metabolic enzymes, reinforcing the concept that metabolic regulation during early development is driven more by substrate partitioning rather than by changes in enzyme abundance.

Collectively, these findings support the concept that metabolic adaptation precedes morphological changes and suggest that early-life alterations in mitochondrial function may contribute to the later in life phenotypes commonly observed at market weight. These results provide further evidence that the metabolic characteristics of HAL and RN mutations are dynamic,

age-dependent, and strongly influenced by muscle metabolic type. Understanding how these mutations shape early metabolic trajectories improves our mechanistic insight into nutrient utilization, muscle growth, and efficiency in livestock, with potential implications for genetic selection and metabolic management strategies aimed at optimizing production performance.

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