Nutritional Strategies to Improve Pig Growth and Performance

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Animal and Poultry Sciences

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September 9, 2020
Blacksburg, VA

Keywords: skeletal muscle, dietary energy, calcium phosphate, dietary protein, heat stress, satellite cells, micro-RNA

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ABSTRACT

Many factors influence the efficiency of muscle growth including genetics, nutrition, and environment. The neonatal period is characterized as a time of rapid growth. Growth rate is reduced during neonatal nutrient restriction possibly due to altered satellite cell activity which can permanently alter growth potential. Therefore, optimal nutrition is important for maximizing the growth potential of the animal. Heat stress leads to changes in digestion and metabolism, thus alters nutrient availability to muscle. Heat stress is a prevalent problem in the agriculture industry resulting in great economic losses due to reduced growth, fertility, and increased morbidity. The use of functional feed additives is a potential strategy to mitigate these negative effects. The objective of this dissertation was to investigate nutritional strategies to improve growth in pigs during key malleable periods. Three nutritional studies were conducted to discern the optimal inclusion levels of calcium phosphate, energy, and protein in the diet to maximize neonatal muscle growth. Adequate dietary calcium phosphate was most efficient for satellite cell function which may be mediated by micro-RNA. Differentiation promoting miR-206 and correspondingly the fusion rate was highest in adequate calcium phosphate diets. Excess protein diets enhanced body and muscle growth, while deficient protein was detrimental to growth. Dietary protein treatments altered energy metabolism genes, and genes regulating protein degradation were upregulated in deficient protein diets. Dietary energy levels did not influence body weight, however feed efficiency improved with energy balance. Excess energy diets had the lowest fusion rates and the lowest differentiation promoting miR-1 expression. These data suggest that nutrient inclusion levels are important for satellite cell function and may mediate satellite cell activity through the

expression of micro-RNAs. The final study sought to discern the ability of supplementation of an artificial high-intensity sweetener and capsicum oleoresin to mitigate the negative effects of heat stress on pig performance. Heat stress leads to increased body temperature and respiration and was detrimental to metabolic flexibility. Supplementation helped improve feed efficiency and maintain metabolic flexibility. These data indicate that supplementation may be an efficient strategy to mitigate heat stress.

Keywords: Satellite Cell, Neonatal Nutrition, Calcium and Phosphorous, Energy, Protein, Heat Stress, Artificial Sweetener, Capsicum Oleoresin, Skeletal Muscle

General Audience Abstract

Muscle is an important tissue to consider when optimizing growing conditions in feed animals due to its function as a consumer good. Many factors influence the efficiency of muscle growth including genetics, nutrition, and environment. Fractional growth rates are highest during the neonatal period and animals require adequate nutrients to facilitate this growth. Nutrient restriction reduces growth rate and can lead to permanent changes the animals' body size and composition later in life. Therefore, optimal nutrition is important for maximizing the growth potential of the animal. While the nutrients in feed can be controlled to improve growth, other factors are more difficult to regulate. Heat stress is a prevalent problem in the agriculture industry resulting in great economic losses due to reduced growth, fertility, and increased morbidity. The use of functional feed additives is a potential strategy to alleviate these negative effects. The objective of this dissertation was to investigate nutritional strategies to improve growth in pigs during key malleable periods. Three nutritional studies were conducted to determine the optimal inclusion levels of calcium phosphate, energy, and protein in the diet to maximize neonatal muscle growth. Satellite cells are muscle-specific stem cells that help facilitate the growth of muscle. Altering the ability of satellite cells to proliferate and fuse impairs the ability of muscle to grow and repair. Adequate dietary calcium phosphate was most efficient for satellite cell function. Excess protein diets enhanced body and muscle growth, while deficient protein was detrimental to growth. Dietary protein treatments altered energy metabolism genes, and genes regulating protein degradation were upregulated in deficient protein diets. Dietary energy levels did not influence body weight, however, feed efficiency improved with energy balance. Satellite cells from excess energy diets had the lowest fusion rates. These data suggest that nutrient inclusion levels are important for satellite cell function and growth. The final study sought to discern the ability of the

supplementation of an artificial high-intensity sweetener and capsicum oleoresin to mitigate the negative effects of heat stress on pig performance. Heat stress leads to increased body temperature and respiration and was detrimental to metabolic flexibility. Supplementation helped improve feed efficiency and maintain metabolic flexibility. These data indicate that supplementation may be an efficient strategy to mitigate heat stress.

Acknowledgements

Throughout my graduate career at Virginia Tech, I have received a great deal of support. I express my sincere gratitude to my advisor, Dr. Rob Rhoads, for giving me this opportunity. I appreciate you providing guidance and an environment to grow as a scientist. Thank you for your insights, laughs, and reminders to stay calm in the face of challenges. Your faith in me has helped propel me through this journey.

I would like to thank the accomplished members of my committee. The opportunity to work with you all has helped to shape me into the scientist I am today. Dr. Johnson, thank you for the opportunity to expand my knowledge on a project outside of this work. Dr. El-Kadi, our many projects have been a highlight of my time here. Dr. Gerrard, thank you for the advice you've provided me and your hallway banter. Dr. Frisard, thank you for your support both in and outside of this project. I look forward to continuing learning from you all in the future.

I would like to acknowledge the past and present members of my lab, especially Dr. Dane Fausnacht, whose help was essential for the completion of this degree. I am grateful to my officemates who have become lifelong friends and made Blacksburg feel like home. Thank you to Sydney Lewis and Kacie Seymore for your camaraderie during many projects. I am grateful for the tremendous help of our undergraduates. I would like to especially thank Alexa Lynn, Rachel Piepenbrink, Ashley Risinger, Joon Choi, and Tom McCabe for their hard work above and beyond what was asked of them.

My biggest thanks goes to my family for supporting me, encouraging me, and believing in me. Words cannot express how deeply grateful I am for all that you have done for me.

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Introduction

Muscle is a multinucleated post-mitotic tissue; therefore, postnatal muscle growth occurs mainly through muscle hypertrophy of existing fibers. In pigs, the majority of muscle fibers are formed in utero, and muscle fiber number is set at birth. The neonatal period is characterized by rapid growth and protein deposition of skeletal muscle (Carr et al., 1978; Davis et al., 1989; Davis et al., 1996). To keep up with the protein demands of the growing fiber, resident muscle stem cells called satellite cells donate their nuclei and ribosomes to existing fiber to increase the capacity for protein synthesis (Fiorotto et al., 2014). Therefore, optimizing growth during the neonatal period through improving satellite cell activity can have lasting impacts on muscle mass and body composition.

Growing organisms exposed to unfavorable conditions adapt to their circumstances. While these adaptions may help with survival, they can have lasting effects that influence subsequent growth and function of the animal (Barker, 2007). Nutrient restriction leads to decreases in growth and composition of growing pigs with the type and severity of nutrient restriction influencing the adaptive responses. While older animals are relatively able to cope with nutrient deficiencies and recover so that they are not different from their unrestricted counterparts, restriction during the neonatal period influences weaning weight which is correlated to altered carcass composition.

Animals undergoing heat stress counter high environmental temperatures with adaptive responses to reduce hyperthermia (Black et al., 1993). However, these responses can have negative consequences to muscle and growth. Heat stress alters body composition and reduces growth rates which are detrimental and costly to producers (Baumgard and Rhoads, 2013a). Therefore, alleviating the negative consequences of heat stress is of importance to animal health and production. Many management strategies have been implemented to lessen the effects of heat

stress. However, these strategies can be costly to producers. Nutritional supplementation has emerged as a potential method to increase nutrient digestion in pigs to improve nutrient availability and growth.

While an abundance of studies demonstrates the importance of nutrition to growth, less is known regarding how nutritional interventions during critical periods such as neonatal growth and heat stress affect muscle growth. This study sought to discern 1) the effects of nutrient inclusion levels on muscle growth in neonatal pigs, and the mechanisms that control these changes, and 2) the impact of supplementation with an artificial sweetener and capsicum oleoresin to mitigate the negative consequences of heat stress.

Chapter 1: Literature Review

Dietary Calcium and Phosphorus

Calcium (Ca) and phosphorous (P) are the two most abundant minerals in the body. The skeleton contains 98% of the bodies calcium in the form of calcium phosphate ($CaPO_4$). The 2% of remaining calcium is found throughout the body in extracellular and cellular fluids and preforms key functions in neuromuscular signaling, metabolism, and enzyme activation (Soares Jr, 1995a; Pond et al., 2005). The skeleton stores 80% of the phosphorous in the body with the remaining 20% serving essential functions including cell membrane composition, metabolism, cell differentiation, and maintaining acid-base buffering (Soares Jr, 1995b). In the cell, phosphorus is a component of nucleic acids, nucleotides like adenosine triphosphate (ATP), phospholipids, and phosphoproteins (Kebreab and Vitti, 2005). Phosphorus is an essential nutrient, however only 40% of P consumed is utilized. Phosphorus reserves are finite so inefficient utilization can lead to depletion of the supply which may be exhausted in as little as 50-100 years (Cordell et al., 2009). The metabolism of Ca and P are interconnected and insufficient or excess concentrations in either mineral will impede efficient utilization and metabolism of the other (Kebreab and Vitti, 2005).

Calcium Phosphate Supplementation

Diets consisting of grain-oilseed lack enough Ca for non-ruminant requirements, and phytates in cereal grains and oilseed meal can reduce the availability of Ca and P. Therefore, Ca and P must be provided as a supplement in non-ruminant diets (Näsi, 1990; Veum et al., 2007; Veum and Ellersieck, 2008). Dietary Ca is mainly supplemented as ground limestone ($CaCO_3$) which is the most abundant source of calcium on earth (McDowell, 2003). Phosphorus is extremely unstable and reactive. Consequently, all P in nature are found as phosphates or orthophosphates (McDowell, 2003). Calcium supplements are found relatively cheap compared to phosphate

supplements, causing the selection of phosphate supplements to be taken under careful consideration to ensure that adequate availability and efficient Ca:P ratio is provided (Hoenderop et al., 2005). Adequate amounts of these minerals are especially important for nursery pigs because of their involvement in skeletal structure development, muscle tissue deposition, and muscle contraction. Because of the economic cost and the environmental concerns, phosphorus has a low safety margin in nursery pig diets. Conversely, calcium levels are typically high in nursery diets due to the low cost of calcium, unaccounted for contributions from carriers, and variability in estimates of concentrations in feed. However, excessive calcium have a pronounced negative effect on growth performance (González-Vega et al., 2016). Diets with excessive calcium and reduced or deficient phosphorous levels have an even greater impact on growth performance (González-Vega et al., 2016; Wu et al., 2018a). To maintain optimal efficiency and animal growth, a Ca:P ratio should be maintained at 1.10:1 to 1.25:1 total Ca to total P ratio or between 1:20 to 1.40:1 digestible Ca to digestible P ratio (González-Vega et al., 2016). Phosphorous requirements as estimated by the NRC in 2012 have emerged to be greater than recommendations of digestible phosphorus as a percentage of the diet (Vier et al., 2017; Wu et al., 2018b). Phytase is the essential enzyme that hydrolyses phytate. Because it is insufficiently secreted in avian and mammals, but is abundant in microbial systems, it is important to provide feed with high P availability (Selle and Ravindran, 2008). Although providing adequate P availability in the diet will ensure adequate P nourishment, it also leads to the potential to have excessive excretion of P, expensive feed, and increased demand for P (Selle and Ravindran, 2008)

Dietary calcium and phosphorous absorption

Many factors affect the absorption of dietary Ca and P from the GIT including concentrations of both Ca and P, the ratio between the two, phytase activity, and the physiological

state of the animals. The majority of Ca and P absorption occurs in the duodenum and jejunum of both ruminants and non-ruminants (Partridge, 1978; Liu et al., 2000; Pfeffer et al., 2005). The low pH (5-6) present in the lumen of the duodenum and jejunum stimulates the ionization and efficient absorption of Ca. In swine, Ca and P are absorbed in small amounts in the caecum, but not in the colon (Liu et al., 2000). To enhance Ca and P absorption in non-ruminants, vitamin D must first be converted to its active form, calcitrol ($1,25(OH)_2D_3$) (DeLuca, 2008) About half the Ca is absorbed by two methods of transport; active and saturable, and passive and non-saturable in the small intestine. When calcium levels are low, the predominant transport of calcium is the active and saturable method. The saturable transport is mediated by calcium binding proteins. One such binding protein, TRPV6, is present in highest concentrations on the apical side of the intestinal epithelium which stimulates calcium uptake into the cell through the formation of a selective calcium channel (den Dekker et al., 2003). Calcitriol stimulates the expression of the TRPV6 gene through the vitamin D receptor. Once inside the cell, Ca is bound to calbindin D_{9k} and transported to the basolateral side membrane where it then dissociates from calbindin and are actively transported out of the cell by the high-affinity membrane Ca^{2+}-ATPase into the interstitial lumen and portal circulation (Bronner et al., 1986). Activity of the vitamin D receptor also regulates the expression of calbindin and activity of membrane Ca^{2+}-ATPase, and therefore, calcitriol is the primary regulator of this Ca transport method (Bringhurst and Leder, 2006). When intraluminal Ca is high, passive, non-saturable movement of Ca is the predominant method to transport Ca across the interstitial lumen through paracellular channels (Karbach, 1992). Endogenous calcium from bile and other digestive juices moves into the interstitial lumen, and their excretion is scarcely affected by dietary or serum calcium levels. The net absorption of calcium from the digestive tract characterizes the differences between active and passive absorption and endogenous intestinal

secretions (Anderson et al., 2016). Animals are able to adjust the efficiency of Ca absorption to meet changes in requirements (Soares Jr, 1995a). Young animals absorb more Ca than mature animals (Hansard and Crowder, 1957; Horst et al., 1978)

Like calcium absorption, phosphorous absorption in the jejunum contains a saturable, active, sodium-dependent absorption that responds to vitamin D and a non-saturable passive paracellular transport. The active transport method on the luminal brush border is stimulated by calcitriol, like Ca, to increase expression of the sodium phosphate transporter (Ontjes, 2011). Without vitamin D basal absorption of phosphate is much higher than calcium indicating that passive absorption is the preferred method (Cross et al., 1990). Endogenous losses of phosphorus increases with the animals weight (Pettey et al., 2006).

Calcium and phosphorous homeostasis and regulation

The movement between calcium stores in bones, inside cells and in extracellular fluid is controlled by parathyroid hormone (PTH), calcitriol, and calcitonin. The parathyroid gland secretes PTH which stimulates the resorption of Ca by the kidneys, from bones into the extracellular fluid, and stimulates production of calcitriol. Calcitriol increases Ca and P absorption by the GIT, as well as stimulation resorption from bones. While calcitriol and PTH increase resorption from bone, calcitonin increases the uptake of Ca into bone. Calcium excretion by the kidneys is inhibited by all three hormones.

The homeostasis of intracellular calcium is tightly regulated by active pumps and exchanges to keep cytoplasmic concentrations at 0.1μM, while extracellular fluids and intracellular compartments like the endoplasmic/sarcoplasmic reticulum (ER/SR) and lysosomes contain much higher concentrations. Opening Ca channels in the plasma membrane or an intracellular store can cause a rapid flux of Ca into the cytoplasm quickly increasing the

concentration of Ca. Closing these channels and using pumps and exchanges to remove Ca from the cytoplasm will quickly reduce the Ca concentrations. However, this is an energy consuming process, and prolonged cytoplasmic exposure to high Ca concentrations can cause toxicity and cell damage with precipitation of $CaPO_4$, and damage to Ca sensitive kinases and phosphatases that control cellular functions (Orrenius et al., 2003).

Calcium and phosphorous in skeletal muscle

In all muscle, Ca is used as the primary regulatory and signaling molecule. In skeletal muscle, contraction and relaxation of muscle is controlled by Ca through the troponin-tropomyosin system that is associated with actin filaments. During the resting state, the intracellular Ca concentration of the myofiber is maintained at ~50nM. Calcium is released from the SR from two channels, the IP_3 receptor (IPR) and the ryanodine receptor (RyR). In skeletal muscle, voltage gated L-type Ca channels are activated when an action potential depolarizes the surface membrane and t-tubules. The L-type Ca channels undergoes a conformation change that directly causes activation and opening of the RyR and release of Ca from the SR to increase local cytosolic Ca concentrations to ~100 fold higher levels. Once released, Ca binds to one of the troponin subunits (troponin C) eliciting a conformational change to allow for crossbridge cycling and initiation muscle contraction. To terminate contraction, the sarco/endoplasmic reticulum calcium-ATPase (SERCA) actively pumps Ca back into the SR, and the muscle relaxes as Ca concentrations decline back to resting levels (Sandow, 1952; Endo, 1977).

In addition to muscle contraction, Ca also regulates the provisions of ATP controlling the energetics of muscle. The stimulation of the sarcolemma results in activation of second messengers, including calcium which binds to intracellular calcium-binding proteins. These binding proteins can act on specific calcium sensitive proteins or indirectly via phosphorylation.

One such, calmodulin activates calcium-calmodulin-dependent protein kinase which in turn phosphorylates the target protein with ATP. It has also been proposed that calcium signaling in muscle may increase glucose uptake and GLUT4 translocation through contraction-stimulated glucose transport (Holloszy and Hansen, 1996).

Deficiencies in PO_4 result in a reduction in muscle and skeletal tissue growth (Cromwell et al., 1995; Jendza et al., 2005). The timing of when these deficiencies occur impacts their severity. Deficiencies in PO_4 early in life are more detrimental to growth than deficiencies later in life. (Driver et al., 2006). During dual Ca and PO_4 deficiencies, PO_4 has a greater impact on muscle and bone growth (Shapiro and Heaney, 2003). Dietary PO_4 has been shown to reduce proliferation of satellite cells in neonatal pigs (Alexander et al., 2012).

Protein Metabolism

Pigs are monogastric animals that derive the protein and energy needed from feed. Protein is a macronutrient essential to growth, but it is an expensive component to swine diets. To maximize profitability, producers reduce protein content. However, the effects of reduced protein can impact growth rate.

Protein Digestion

Dietary protein digestion begins in the stomach with the protease pepsin by hydrolyzing protein's peptide bonds into smaller polypeptides. Pepsin partially digests 10-15% of dietary protein (Binder and Reuben, 2005; Devlin, 2006). The partially digested protein then enters the small intestine where the pancreas secretes pancreatic protease and bicarbonate to neutralize stomach acid and bring the pH to a more optimal level for protease activity. Pancreatic proteases trypsin, chymotrypsin, elastase and carboxypeptidases continue to cleave polypeptides into oligopeptides and amino acids. Oligopeptides are further cleaved one amino acid (AA) at a time

by exopeptidases, resulting in protein digestion into 30% free amino acids and 70% oligopeptides 2-8 amino acids in length (Binder and Reuben, 2005). Transport proteins on intestinal enterocytes facilitate amino acids and di- and tripeptides across the brush border. Some of the di- and tripeptides are then hydrolyzed within the cells to free AA by intracellular peptidases. Free amino acids enter circulation through the basolateral membrane and can be taken up by other tissues (Binder and Reuben, 2005).

Protein in muscle

In skeletal muscle, protein serves as building blocks for contractile proteins and as a source of energy in situations where other energy sources are not available. Muscle growth occurs when the rate of protein synthesis is greater than the rate of degradation. Muscle mass and protein synthesis is mainly regulated through the insulin-like growth factor -1 (IGF1)- protein kinase-b (AKT)/ mammalian target of rapamycin (mTOR) pathway. Signaling begins with the IGF1 ligand binding to its receptor, which causes recruitment of insulin receptor substrates that activate phosphoinositide 3-kinases (PI3K) to produce phosphatidylinositol-3,4,5 triphosphates (PIP3). PIP3 activates AKT proteins which phosphorylates mTOR rendering it active. Activated mTOR complexes with the protein regulatory associated protein of mTOR complex 1 (RPTOR) to form mTOR complex 1 (mTORC1), and RPTOR independent companion of MTOR complex 2 (RICTOR) to form mTOR complex 2 (mTORC2). The formation of mTORC1 results in the positive regulation of genes involved in protein translation and synthesis, and the negative regulation of inhibitors of protein synthesis (Schiaffino and Mammucari, 2011). Increased activation of this pathway produces increased muscle hypertrophy.

Leucine stimulates protein synthesis through activation of the translation process. Amino acid depletion and starvation results in the inhibition of the translation initiation complex through

eukaryotic initiation factor 4E (eIF4E) being bound and inactivated by eiF4E binding protein (4EBP1). Leucine activates mTORC1 kinase which then phosphorylates 4EBP1 leading to active eIF4E allowing for translation and protein synthesis (Ribeiro et al., 2015).

Protein impacts on animal growth

For decades, the environmental impact of swine production has been a concern (Carpenter et al., 2004). Excess dietary protein in the diet is excreted in the urine and feces as nitrogen. A portion of this nitrogen is emitted into the atmosphere when it is oxidized to nitrous oxide (N_2O) contributing to greenhouse gasses. It has been suggested that a 1% reduction in crude protein (CP) by substituting with crystalline amino acids would reduce excreted nitrogen in feces by 8% (Kerr and Easter, 1995). However, providing too little dietary protein leads to deficits in growth (Hansen and Lewis, 1993; Chen et al., 1999).

Growing pigs require differing levels of dietary CP, amino acids (AA), and nitrogen (N) to meet the physiological demands for maintenance and growth (Escobar et al., 2004; Humphrey and Klasing, 2004). Increasing CP levels has been associated with improvements in average daily gain (ADG) (Hansen and Lewis, 1993; Chen et al., 1999). Protein requirements start at 26% crude protein (CP) in young pigs and decrease with age. Low protein diets are beneficial in swine due to a reduction in nitrogen secretion, hindgut fermentation, and diarrhea, and are thus advantageous to the swine industry as a strategy for improving efficiency. In piglets, reducing CP by 55% reduced nitrogen excretion but did not have detrimental effects to performance and body composition (Le Bellego and Noblet, 2002). However, low protein diets can also lead to altered carcass composition with increased adipose tissue and decreased lean mass (Kerr et al., 1995). Pigs fed a high amino acid diet in the grower phase had increased growth rate and less backfat than pigs on a low amino acid diet (Chiba et al., 2002). Young pigs will exhibit a period of compensatory

growth following a period of dietary restriction that offsets their deficit so they are not different from their counterparts (Chiba, 1995). However, the ability of the pig to engage in compensatory growth is limited by the diets provided after restriction. Many factors influence the degree and longevity of the response such as severity of the restriction, duration, stage of growth, and composition of other nutrients. In fact, Low CP diets improved weaning pig growth performance when the diet contained a high metabolizable energy level (Fang et al., 2019).

Energy Metabolism

Energy is the principle driver of metabolism. Without sufficient energy, dietary protein is diverted towards energy production instead of converted into body protein which negatively impacts growth. Animals must consume sufficient energy to support the demands of the body for maintenance, growth, and tissue function. Free energy in muscle is required for protein synthesis and degradation. Alterations to energy supply may affect protein deposition rate, and changes in the rate protein turnover will change energy requirements (Siems et al., 1984; Summers et al., 1988). Metabolizable energy intake increases protein synthesis rate in muscle beyond protein degradation rate.

Muscle tissue derives energy from both extra- and intra-muscular fuel sources. Extra-muscular fuel sources include glucose, lactate, non-esterified fatty acids (NEFA), volatile fatty acids (VFA), triglycerides (TG), and ketone bodies. Intra-muscular fuel sources consist of glycogen and TGs. The utilization of these fuel sources mainly depends on their availability and the energy demands of the cell, however, is also influenced by the animal's activity and enzyme activity. The two main fuel sources utilizes by skeletal muscle to generate ATP are carbohydrates and fat (Romijn et al., 1993).

Glucose metabolism

Dietary carbohydrates are hydrolyzed in the small intestine to monosaccharides like glucose or galactose where they cross the brush-border membrane through the sodium glucose transporter SGLT1. To enter into the blood stream, glucose is transported out of the enterocyte through GLUT2 (Röder et al., 2014). In circulation, glucose is transported to other tissues to be catabolized for energy.

Skeletal muscle consumes the most glucose in the body, however glucose fractional extraction rates average 9% in growing pigs (Wray-Cahen et al., 1995) as a result of lactate release from muscle Wray-Cahen et al., 1995). Lactate is also taken up for oxidation or glycogenesis in oxidative fibers (McCullagh et al., 1996). Glucose transport into muscle fibers is facilitated by glucose transporter 4 (GLUT4). This receptor resides inside the cell during basal conditions where upon stimulation is rapidly transported to the cell surface (Aschenbach et al., 2009). Once inside the muscle fiber, glucose can follow many metabolic pathways depending on the needs of the cell. Glucose may undergo direct oxidation, glycolysis to L-Lactate to be released back into circulation, or glycogen synthesis when the energy demands of the cell are met and is the major fate of glucose (Hocquette et al., 1998). Low ATP or lactate in the cell initiates glycolysis through activation of 6-Phosphofructo-1-kinase (PFK) (Regen et al., 1964). Aerobic oxidation of glucose is regulated by the pyruvate dehydrogenase (PDH) complex which is inhibited by acetyl-CoA and ATP. Glucose uptake is stimulated by insulin for storage and oxidation by stimulating GLUT4, glycogen synthase and PDH.

Lipid metabolism

Dietary lipids are initially digested in the stomach through lipase activity and continue in the small intestine. Lipase activity degrades fatty acids to produce free fatty acids and 2-

monoglycerides (Bach and Babayan, 1982; Carey et al., 1983). Medium-chain fatty acids are broken down into either a glycerol backbone with 3 fatty acids or absorbed intact into the small intestine and transported to the liver through the portal vein. Long chain fatty acids are emulsified with bile salts and cross into enterocytes to be converted back into TGs. Triglycerides are then incorporated into chylomicrons and enter the lymphatic system (You et al., 2008).

Oxidative metabolism utilizes both lipids and products of glycolysis and serves as the primary source of energy production for sustained energy demands (Eaton et al., 1996). Lipoprotein lipase (LPL) hydrolyzes TG from circulating lipoproteins generating NEFAs to be taken up and metabolized by tissues. Uptake of NEFA into hindlimbs is linearly correlated to arterial concentrations. Fatty acids can be partially digested to form acyl-CoA by peroxisomes and transported into the mitochondria by the rate limiting carnitine palmitoyl transferase I (CPT I). Within the mitochondria, fatty acids are converted by -oxidation enzymes into acetyl-CoA which then enters the citric acid cycle. The by-products of fatty acid oxidation and the citric acid cycle are then used by the electron transport chain to generate ATP (Kunz, 2001).

Energy impacts on animal growth

Dietary energy concentration can impact feed intake as high energy levels can decrease feed intake, while low energy level can decrease the deposition of protein. Protein deposition and intake is related to the concentration of energy provided. It is difficult to determine the energy requirements of young pigs because of their varying average daily feed intake (ADFI) and physiological stressors like weaning. From birth to 56 days of age, piglets have low digestive enzyme activity for protein and energy. Therefore much of the provided protein and energy cannot be digested (Jensen et al., 1997).

In general, prolonged periods of negative energy balance results in a decrease in total body mass. While the major change in body composition due to restricted energy is loss of fat, piglets at birth lack fat deposits and have a high demand for energy (Pastorelli et al., 2009). Therefore, negative energy balance can be especially detrimental to piglets. Well-fed piglets synthesize TGs rapidly results in a body-lipid increase from 1% to 14% in 2 weeks (Wu et al., 2004). Early nutrient restriction in chickens causes reduced growth that results in a lower body weight later in life (Palo et al., 1995). Nutrient restriction reduces satellite cell number and activity as well as decreases the expression of myogenic genes and reduces muscle mass (Halevy et al., 2000; Halevy et al., 2003; Jeanplong et al., 2003). In weaning pigs, a decrease in dietary energy level increased feed intake and decreased efficiency, which is costly to producers (Fang et al., 2019). Conversely, high energy diets also can lead to an increase in adipose tissue and altered body composition which leads to an undesirable carcass. In piglets, high energy density diets impair intestinal function and influence amino acid utilization which reduces weight gain at weaning (Adebowale et al., 2019). Reduced weaning weight is associated with a decrease in carcass quality and thus reduces profitability. There is an increasing body of evidence indicating that these changes to growth and composition due to altered nutrition are a result of altered satellite cell function (D'Souza et al., 2015; MacGhee et al., 2017). A low plane of nutrition in newborn calves resulted in reduced growth and altered satellite cell activity (MacGhee et al., 2017).

Satellite Cells

Satellite cells are the resident stem cell population in muscle that reside beneath the basal lamina but outside of muscle fibers (Mauro, 1961). Satellite cells constitute ~1% of the nuclei in muscle (Lepper et al., 2011; Yin et al., 2013b). These satellite cell populations remain quiescent until activated in response to damage, overuse, mechanical stretch or growth factors where they

will then begin to proliferate (Webster et al., 2016). Satellite cells have the ability to differentiate and fuse, as well as to self-renew to maintain a stable stem cell population despite multiple rounds of regeneration stimulus (Wang and Rudnicki, 2012). Through this process, nuclei accumulate in muscle fibers allowing for increased protein synthesis and muscle hypertrophy.

Satellite cell quiescence

Quiescent satellite cells express the satellite cell marker Paired Box 7 (Pax7), a transcription factor that maintains quiescence and inhibits differentiation. (Seale et al., 2000; Olguin and Olwin, 2004). Satellite cell quiescence is an active process (not a default state) which occurs through the activation of NOTCH and WNT inhibition of myoblast determination protein 1 (MyoD) (Bjornson et al., 2012). Additionally, myogenic genes are transcribed and sequestered rapidly (Hausburg et al., 2015).

Satellite cell activation

Activation of satellite cells is stimulated by hepatocyte-growth factor (HGF) which is localized in the extracellular domain of uninjured skeletal muscle fibers (Allen et al., 1995; Tatsumi et al., 1998). When HGF is released from its sequestered location in the extracellular matrix, it binds to the intracellular signaling c-mesenchymal–epithelial transition receptor (Tatsumi et al., 2001). In order to catalyze HGF release, matrix metalloproteinases are activated by nitric oxide synthase- mediated nitric oxide production (Yamada et al., 2008).

Upon receiving environmental signals to activate, satellite cells enter the cell cycle and proliferate characterized by the expression of PAX7 and MyoD, MyoG, and Myf5 expression (review by (Yin et al., 2013b). Once activated, satellite cells phosphorylate p38α/β which phosphorylates MK2 resulting in phosphorylation and inhibition of tristetraprolin (TPP). By inhibiting TPP, which functions to degrade mRNA, MyoD is stabilized allowing for its translation

and function as a myogenic transcription factor (Cooper et al., 1999; Jones et al., 2005). After expansion, myoblasts begin terminally differentiating to fuse with existing fibers through down regulation of MYF5 and later MyoD. A subset of satellite cells will return to quiescence to self-renew and maintain the satellite cell pool (Kuang et al., 2007).

Satellite cells incur metabolic fluctuations as their cell fate changes. Differentiation leads to alterations in mitochondria, glucose homeostasis, protein metabolism, and redox signaling (Fulco et al., 2003; Fulco and Sartorelli, 2008) It has been proposed that satellite cell activation from quiescence to an activated state occurs due to metabolic changes in lipid homeostasis, mitochondrial activity and ATP production. As satellite cells become activated, their metabolism shifts away from fatty acid and pyruvate oxidation towards glycolysis. (Fukada et al., 2007; Ryall, 2013).

MicroRNA in Skeletal Muscle

Increased skeletal muscle development early in life can lead to an increased growth potential of the animal. Postnatal muscle growth has been characterized as a hypertrophic event because fiber numbers are determined prenatally and become fixed around the time of birth. However, during postnatal growth muscle fibers display a substantial increase in DNA content such that muscle accumulates between 50-99% of the total DNA after birth depending on the species and muscle type (Allen et al., 1979). Nuclei within muscle fibers are post-mitotic and the postnatal accumulation of DNA is due to the division and fusion of satellite cells (skeletal muscle stem cells) to myofibers (Moss and Leblond, 1971). The expression of muscle-specific regulatory factors controls myogenic progression of satellite cells, via the reprograming of gene expression. MicroRNAs (miRNA) function to regulate these genes at the post-transcriptional level to control cell proliferation, differentiation, and lineage (Friedman et al., 2009). The incorporation of

miRNAs by myogenic transcription factors expands the complexity and precision in gene regulation of muscle development. Identifying the role myogenic miRNA's play in the regulation of skeletal muscle development will enhance the understanding of growth and provide the basis for future therapies.

MicoRNA Processing

MicroRNA (miRNA) are a family of short single stranded noncoding RNA, ranging in length from 18-25 nucleotides (Friedman et al., 2009). These miRNA function to inhibit translation or promote messenger RNA (mRNA) degradation by binding to target mRNA with complete or partially complete base pairing (Hamilton and Baulcombe, 1999; Reinhart et al., 2000; Krol et al., 2004). A single miRNA has the ability to bind to several hundred mRNAs, and conversely a single mRNA can be bound by several different miRNAs (Friedman et al., 2009). An increase in a specific miRNA would consequently result in a decrease in protein product. Since a miRNA can bind to several targets, the consequence is not a one-to-one relationship between an increase in miRNA and a decrease in protein (Wang, 2013). Therefore, miRNAs function to fine tune gene expression patterns.

Both mRNA and miRNAs are transcribed from genomic DNA in the nucleus. First, miRNA is transcribed as primary miRNA (pri-miRNA) characterized as several kilobases long (Bartel, 2004) and mostly from independent transcripts. However, some pri-miRNAs originate through RNA splicing (Yang and Lai, 2011). The expression of miRNA is regulated through their promoter regions, and transcriptional regulation of an individual miRNA can be controlled through numerous different transcription factors. Some transcription factors have the ability to bind to pri-miRNA rather than miRNA promoter and affect further processing (Goljanek-Whysall et al., 2012) including SMAD proteins, which regulate TFGβ/BMP signaling, that have been shown to bind to

a range of pri-miRNAs to control their processing (Davis et al., 2008; Davis et al., 2010). Approximately one third of miRNAs are imbedded in introns of protein-coding genes and processed after splicing (Van Rooij and Olson, 2007). The long pri-miRNA undergo cleavage still in the nucleus to form a 60-70 nucleotide intermediate called pre-miRNA by the nuclear protein DiGeorge critical region 8 (DGCR8). This DGCR8 protein recognizes the sequence and the ribonuclease Drosha cuts the pri-miRNA and leaves a 5' phosphate and a two-nucleotide overhang on the 3' strand. The activity of Drosha provides another level of regulation by being dependent of the sequence of the pri-miRNA so that not all are processed with equal efficiency (Feng et al., 2011a; Feng et al., 2011b). The cleavage by Drosha results in the formation of miRNA transcripts that fold back on themselves to produce a hairpin structure that can be transported out of the nucleus to the cytoplasm through the export receptor Exportin-5 (Lee et al., 2003). In the cytoplasm, the hairpin structured pre-miRNA is further processed by the RNase Dicer to excise the miRNA from the hairpin stem, which yields a ~22 nucleotide duplex miRNA (Lee et al., 2003; Friedman et al., 2009). This double stranded miRNA is unwound by helicase before one strand, often the one with the less thermodynamically stable 5' end, is transported into the RNA induced silencing complex (RISC) (Schwarz et al., 2002; Khvorova et al., 2003).

RNA Silencing

Once formed, the mature miRNA is incorporated into a RISC, which is a multiprotein complex that functions to prevent translation or promotes mRNA degradation (Figure 1.1). The RISC includes an Agronaute protein, that binds the miRNA to facilitate mRNA recognition. Argonaute proteins have distinct activities and expression patterns which provide another level of regulation in the miRNA pathway by determining their specificity and functionality (Czech and Hannon, 2011). The miRNA guides the RISC to the 3' untranslated region (UTR) of the target

mRNA. Nucleotides 2-8 of the miRNA, called the seed region, will bind to the target mRNA with complete or partially complete base pairing (Doench and Sharp, 2004; Lewis et al., 2005). Due to

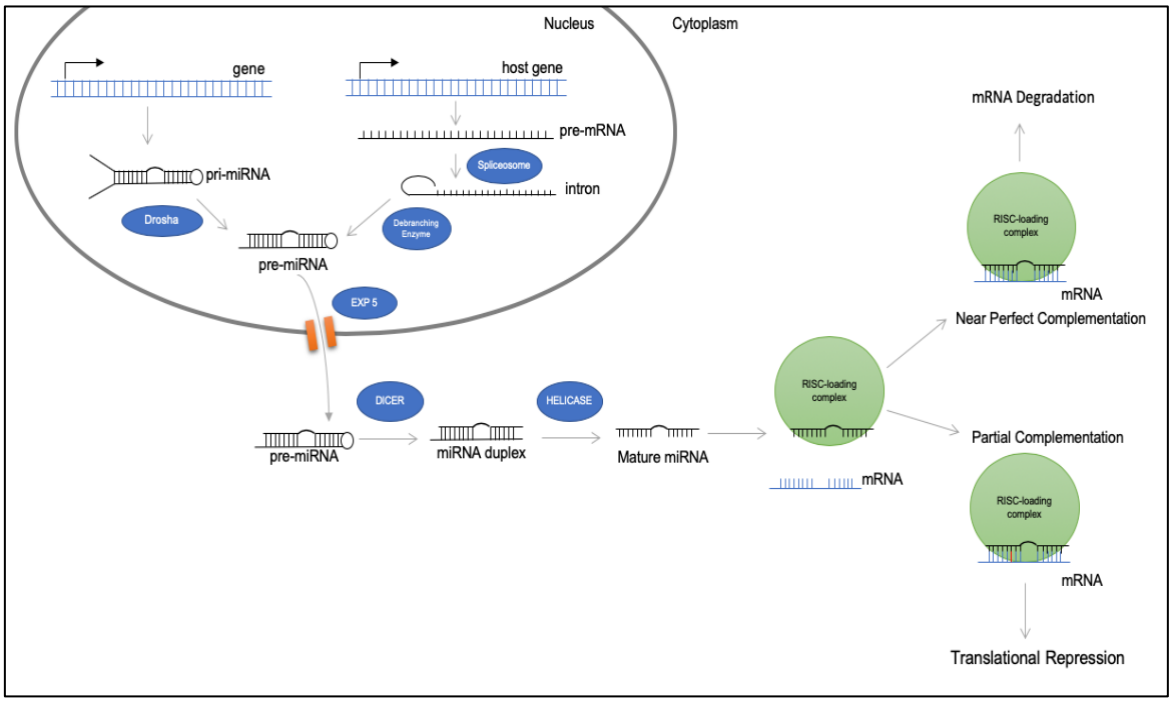


Figure 1.1 Biosynthesis of miRNA and miRNA mechanism of action

imperfect pairing each miRNA is estimated to regulate about 200 targets (Lewis et al., 2003), and an estimated 60% of mRNAs may be regulated by one or more miRNAs (Friedman et al., 2009). Once bound to the target mRNA, the RISC complex will either degrade the mRNA if there is near-perfect base pairing between the guide miRNA and the target mRNA, or will repress translation if there is imperfect base pairing. Direct cleavage of the mRNA is performed by the agronaute protein which cleaves the target nucleotides that pair with the 10^{th} and 11^{th} bases on the guide miRNA (Yuan et al., 2005). Translational repression occurs though inhibition of translation initiation by blocking the pre- initiation complex on the target mRNA, degrading the poly-A tail, or by directing target mRNAs to P-bodies, which are inactive structures that store or degrade mRNA (Behm-Ansmant et al., 2006; Eulalio et al., 2008; Iwasaki et al., 2009). Individual miRNAs do not have

the ability to act alone in degradation or inhibition of translation of mRNA, nor do they target one single mRNA.

Expression of miRNA in Skeletal Muscle

Skeletal muscle development begins with cells from somites committing to the myogenic lineage, proliferating, then terminally differentiating and fusing into multinucleated myofibers. Recently, miRNAs have emerged as key factors in the regulation of muscle development. A loss of function mutation in Dicer in mice is embryonically lethal by day 7.5 (Bernstein et al., 2003). The deletion of a conditional dicer allele in skeletal muscle was created to circumvent the lethality associated with the total deletion of Dicer. These transgenic mice die perinatally and exhibit decreased skeletal muscle mass due to hypoplasia, increased apoptosis of muscle cells, and abnormal myofiber morphology (O'Rourke et al., 2007). Due to Dicer's control of miRNA maturation, these observations support a key role for miRNA muscle development.

Several key processes in muscle tissue are governed by miRNAs such as myogenesis, fiber type shifts, muscle growth as well as atrophy. This group of miRNA's that are enriched in cardiac and/or skeletal muscle are called MyomiRs (McCarthy, 2011). While myomirs are enriched in muscle, some may be found in other tissues and can be considered either muscle exclusive or non-exclusive depending on the tissue and function. MyomiRs are members of the miR1/206 and miR-133 families and include three of the most studied miRNAs being miR-1, miR-133, and miR-206. These three miRNA's are expressed from three chromosomal loci as bicistronic transcripts (Chen et al., 2006). Mirco RNA-1 and miR-133 are clustered together on the same chromosome and transcribed together with post transcriptional modification dictating the production of either miRNA (Chen et al., 2006). Both miR-1/miR-133 are specifically expressed in cardiac and

skeletal muscle tissues, but were absent in other tissues examined (Lagos-Quintana et al., 2002). The expression of miR-206 and miR-133b are expressed specifically in skeletal muscle and is thought to be controlled by an upstream regulatory region that is enriched for MyoD binding (Rao et al., 2006). Micro RNA-1 and miR-206 differ from each other by four nucleotides outside the seed region, and share many gene targets (Dey et al., 2011).

The Role of miRNA in Muscle Development

Myogenic miRNAs regulate fundamental aspects of muscle development. Myoblast proliferation is increased by miR-133a which inhibits serum response factor (SRF), a transcription factor that regulates many genes involved in cell cycle, growth, and differentiation (Chen et al., 2006). The interaction of miR-133a and SRF establishes a feedback loop in which SRF upregulates the expression of miR-133a causing increased repression of SRF and increased proliferation. Repression of miR-133 in mice enables satellite cells to differentiate into brown adipose tissue (Trajkovski et al., 2012; Yin et al., 2013a). In satellite cells, miR-133 maintains satellite cells to the myogenic lineage by inhibiting Prdm16, a zinc-finger transcription factor that is necessary and sufficient to establish the brown adipose lineage (Seale et al., 2007). Conversely, overexpression of miR-133 in preadipocytes causes a reduction in brown adipose specific thermogenic gene expression (Yin et al., 2013a).

The primary function of miR-1/206 is to promote the differentiation of mesodermal and muscle lineages. Mutations introduced into the miR-1 seed sequence abolishes its ability to activate myogenic gene expression (Chen et al., 2006). Myoblast differentiation is stimulated by miR-1/206 through targeting follistatin and utrophin, which both act to maintain myoblasts in proliferative states (Rosenberg et al., 2006). Differentiation is also promoted through miR1/206 inhibition of histone deacetylase 4 (HDAC4) (Chen et al., 2006). Histone deacetylase 4 is a

transcriptional repressor of muscle gene expression, mainly by inhibiting an essential muscle transcription factor, MEF2C (Lu et al., 2000). Thus, by inhibiting HDAC4 with miR-1, MEF2C transcription factor activity is increased which leads to myocyte differentiation. Differentiation is increased by miR-206 through downregulation of the helix-loop-helix protein Id, which represses MyoD. The inhibition of the helix-loop helix protein Id through miR-206 enhances MyoD expression and promotes myogenic lineage differentiation (Kim et al., 2006).

Other miRNAs that are not in the myomiR family play a role in muscle development through regulation of the cell cycle and genes controlling proliferation and differentiation. One such is miR-24 which promotes differentiation but is negatively regulated by TGF-β (Sun et al., 2008). TGF-β inhibits satellite cell differentiation through repression of transcriptional activity of the muscle regulator factors (MRF) family. The MRF family includes MyoD, myogenin, Myf5, and MRF4, and bind to regions upstream of myomiRs to regulate their expression (Rao et al., 2006; Sweetman et al., 2008). Signal transduction of TGF-β promotes retention of Smad proteins 2 and 3 in the nucleus in complex with Smad 4 to control the expression of target genes. One such target is HDAC4, a negative regulator of myogenic commitment by repressing MRF's and is regulated by miR-206 and miR-29 (Winbanks et al., 2011). TGF-β downregulates five miRNAs that are involved in the transcription of myogenic differentiation genes; miR-1, -22, -29, -133, and -206. TGF-β suppresses the levels of miR-206 and -29 to relieve translational suppression of HDAC4 to inhibit differentiation. Increasing the levels of miR-206 and -29 can then work against TGF-β and prevent HDAC4 from repressing differentiation (Winbanks et al., 2011) thus increasing the progression through the myogenic lineage. TGF-βI is targeted by miR-21. During prenatal development in pigs, miR-21 is expressed in skeletal muscle tissue and has been found affect the activity of PI3K/Akt/mTOR signaling by targeting TGF-βI. The expression of miR-21

and TGF-β1 are negatively correlated suggesting that miR-21 targets TGF-β1 to regulate muscle development (Bai et al., 2015). This high level of crosstalk is important to understand the mechanism of action of satellite cells progressing through their cell lineage.

miRNA in Muscle Growth and Function

Postnatal muscle growth and muscle regeneration is dependent upon satellite cells (Buckingham, 2007). Satellite cells are characterized by their expression of Pax7, which is required for satellite cell survival, proliferation and the prevention of differentiation (Relaix et al., 2006). Satellite cell proliferation is inhibited, and myoblast differentiation is promoted by miR-1/miR-206 targeting of Pax-7 (Dey et al., 2011). During satellite cell differentiation, miR-1 and miR-206 are greatly upregulated but become downregulated during muscle regeneration. Additionally, premature overexpression of miR-1/miR-206 inhibits proliferation of satellite cells and increases their differentiation due to Pax7 targeting (Chen et al., 2006). The action of Pax7 is also mediated by miR-486, which acts as a regulator of satellite cell quiescence by suppressing an oncogene, Dek, that is as asymmetrically inherited by the more differentiated daughter cell (Cheung et al., 2012). When satellite cells are induced to differentiate, Pax7 is required for the initial activation of Myf5 and MyoD. Activation of MyoD stimulates miR-1 and miR-206, which represses Pax7 and initiates terminal differentiation into myocytes (Chen et al., 2006).

MyomiR transcription is controlled by many factors like aging or disease, damage, level of physical activity, and nutrition (Drummond et al., 2008; Drummond et al., 2009; Nielsen et al., 2010; Drummond et al., 2011). In adult skeletal muscle, miR-206 has been proposed to promote myogenesis by downregulating tissue inhibitor of metalloprotease 3 (TIMP3), which acts as a repressor of myogenesis. Downregulation of TIMP3 allows for the expression of myogenic genes through the upregulation of TNFα and activation of MAPK (Liu et al., 2010).

Insulin-like Growth Factor-1 (IGF-I) regulates muscle growth and development and acts as both a target and a regulator of miR-1. A feedback loop exists between IGF and miR-1 in which IGF-I binding to its receptor activates signaling pathways that lead to the activation of AKT. Active AKT phosphorylates and inhibits the transcription factor Foxo3a, that works to stimulates the expression of miR-1 (Elia et al., 2009). Furthermore, miR-206 targets the 3'UTR region of IGF-I, and reductions in miR-206 levels causes an increase in IGF-I levels (Yan et al., 2013). The receptor for IGF-I (IGF-IR) had a conserved binding site for miR-133 in the 3'UTR. Increasing miR-133 in C2C12 cells suppresses IGF-IR protein levels at the post-transcriptional level (Ge et al., 2011), therefore decreasing the activation of AKT and the IGF/PI3K/AKT pathway.

Another role of miRNA in muscle is their function in the differentiation of muscle fiber types. At least three slow and cardiac myosin heavy chain (MyHC) genes have miRNAs encoded in their introns that control muscle myosin content, myofiber identity and muscle performance (Van Rooij et al., 2009). The intron of *Myh7*, which encodes the β-myosin heavy-chain protein, includes the miR-208b, and miR-499 is derived from the intron of the *Myh7b* gene. Expression of the miRNA is directly related to the expression of the host gene, so miR-208 is enriched in slow-twitch type I muscles. These miRNAs regulate the expression of transcription factors that repress the slow twitch fiber type such as *Sox6 (Hagiwara et al., 2007; Van Rooij et al., 2009)*. A feedback loop is formed where miR-208b regulates the expression of Sox6, which in turn represses *Myh7* expression, which is the host gene of miR-208b. Thus, these miRNAs work by inhibiting the repressor and promoting the slow twitch phenotype. Inactivation of these miRNAs cause a reduction in Type I fibers and a decrease in β-MyHC expression and an increase in *Sox6* expression in the soleus muscle of mice (Van Rooij et al., 2009). Over expression of miR-499 in the soleus leads to a complete conversion to Type I fibers (Van Rooij et al., 2009).

Micro RNAs may be an important target to improve the performance in agricultural species by playing critical roles in skeletal muscle hypertrophy. Myostatin, a negative regulator of muscle mass, has target sites for several miRNAs. Both miR-208a and miR-499 are capable of reducing the expression of myostatin (Callis et al., 2009; Bell et al., 2010). Furthermore, myostatin may in turn regulate the expression of miRNAs as it has been shown that myostatin null mice have increased expression of miR-133a, -133b, -1 and, -206 (Rachagani et al., 2010). Texel sheep are known for their enhanced muscle mass due to a mutation in myostatin that causes a single nucleotide polymorphism in the 3' UTR which creates a target site for miR-1 and miR-206 (Georges et al., 2006). This causes the translational repression of myostatin, which increases muscle hypertrophy contributing to the muscular phenotype of Texel sheep. Therefore, miRNAs play a key role in the development and growth of skeletal muscle by controlling the expression of myogenic genes.

Micro RNAs play a key role in the regulation of genes involved in every aspect of skeletal muscle development. Proper skeletal muscle development does not occur in the absence of miRNA, resulting in perinatal lethality and altered muscle morphology. Myoblast differentiation is enhanced by miR-1 and miR-206, while proliferation is promoted by miR-133. Skeletal muscle hyperplasia is particularly critical during development, as increased muscle fiber number can increase the growth potential of an animal. Identification of the role and regulation of miRNA in skeletal muscle development will enhance the understanding of skeletal muscle biology which may allow for improvements in the efficiency of meat production, as well as lead to new treatments for muscle related diseases.

Heat Stress in Animal Agriculture

Animal agriculture experiences great economic losses with animals undergoing heat stress, as animals will divert energy from productive mechanisms into adaptive mechanism (Nardone et al., 2010). This leads to reduced growth rates (Hahn, 1999; Marai et al., 2007), decreased fertility (Hennessy and Williamson, 1984; Nardone et al., 2006; Hansen, 2009), as well as increased morbidity and mortality (D'Allaire et al., 1996; Bishop-Williams et al., 2015). A push for productivity in agriculture has led to industry wide increases in growth rates, milk yield, and egg production, however, heat stress challenges these developments by reducing efficiency as nutrients are diverted toward lowering body temperature (Baumgard and Rhoads, 2013b). Almost two decades ago, losses associated with heat stress were estimated to be greater than $300 million (St-Pierre et al., 2003). With ever increasing global temperatures since that time, and a push for raising animals in subtropical environments as demand for animal products rise (Delgado, 2005), losses today would be much greater. Economic losses to the United States swine industry during the summer months, despite attempts to alleviate heat stress, were estimated to be $900 million (Pollman, 2010). Heat stress is a seasonal problem in temperate climates but a consistent challenge in tropical and subtropical environments where it presents as the greatest limiting factor of production efficiency (Renaudeau et al., 2008).

Elevated environmental temperatures cause high thermal loads in humans and livestock that can lead to acute, chronic, and lethal heat related disorders such as heat stress (Wilkins and Wheeler, 2004). Homeothermic animals maintain a thermoneutral core body temperature within a narrow range in which cellular processes and enzymatic reactions function optimally and energy expenditure is minimal. Outside of this range total heat load exceeds the capacity for heat dissipation, reactions slow and high temperatures may cause a breakdown in proteins and cell

death (Wilkins and Wheeler, 2004; Bernabucci et al., 2010). When an animal is unable to dissipate excess heat effectively, heat stress occurs. The effect of elevated temperatures is further aggravated by high humidity because of a reduced capacity for evaporative heat dissipation (Marai et al., 2007). Environmental conditions including air temperature, solar radiation, air flow, and relative humidity exceeding the animals thermoneutral zone limits cause core body temperature to increase. While animals have the ability to adapt to climatic stressors, the response mechanisms used are often detrimental to the productivity and growth (Pragna et al., 2018).

Adaptive mechanisms and consequences to heat stress

Heat stress responses are largely conserved across species and lead to physiological adaptions. Heat stress causes increases in core body temperatures, decreases in growth rates, reproductive efficiencies, and overall health. During heat stress both male and female reproductive functions are negatively impacted causing decreased pregnancy rates, increased embryonic losses, and sperm abnormalities (Hansen, 2009). Heat stress causes decreased milk production and consequently offspring growth rates decline (Black et al., 1993; West, 2003). Pigs are especially susceptible to heat stress due to a lack of functional sweat glands with heat regulation occurring mainly by respiration through evaporative cooling (Ingram, 1965; Curtis, 1983). Pigs also have a relatively large layer of subcutaneous fat that contributes to less effective heat dissipation and a less tolerant heat response. As a result, heat tolerance in pigs occurs primarily through behavioral changes (Bernabucci et al., 2010). Modern strains of pigs have undergone genetic selection towards increased lean mass and greater reproductive potential at the cost of increased metabolic rate and elevated endogenous thermogenesis thus increasing their susceptibility for heat stress related conditions (Nienaber et al., 1997; Baumgard and Rhoads, 2013b).

Heat dissipation during heat stress

Respiration rate increases during hyperthermia, which is used to increase evaporative cooling through the mouth. Excess heat is expelled by vaporizing more moisture through respiratory and cutaneous cooling mechanisms (Berman, 2006). Blood hemoglobin increases with environmental temperature in order to accommodate for the increased oxygen circulation due to the increased respiratory rate (Haque et al., 2013; Okoruwa, 2014). Radiant heat dissipation through the skin is assisted by a repartitioning of blood from splanchnic organs towards peripheral blood flow by vasoconstriction of the gastrointestinal tract (Lambert, 2009). Consequently, reduced blood and nutrient flow compromises intestinal epithelium integrity and function (Yan et al., 2006; Pearce et al., 2013a; Pearce et al., 2013c). Weakened intestinal integrity leads to increased permeability associated with changes in nutrient digestibility and absorption as well as endotoxemia, hypoxia, and inflammation (Hall et al., 2001; Pearce et al., 2013b). While increased respiratory rates and radiant heat dissipation are used to increase cooling, other adaptive mechanisms are used to decrease heat production.

Behavioral adaptions in pigs

Behavioral changes occur in response to heat stress to minimize heat production such as a reduction in locomotion to decrease mechanical heat production, and a voluntary reduction in feed intake to reduce the thermic effect of feed and metabolism (Renaudeau et al., 2008; Baumgard and Rhoads, 2013a). In pigs, reduced feed intake is observed at temperatures above 23.9^{o}C (Kouba et al., 2001). Heat stress causes an upregulation in leptin and adiponectin, adipokines that regulate the hypothalamic axis to reduce feed intake (Hoyda et al., 2012; Morera et al., 2012). Duration of heat stress has an effect on feed intake in which acute heat stress causes a greater reduction than chronic heat stress; 64% vs 36% reduction respectively (Qu and Ajuwon, 2018). This reduction in

feed intake reduces growth and reproductive performances impairing profitability (Nienaber et al., 1997).

Gastrointestinal effects

A consequence of heat stress is the shunting of blood away from splanchnic organs towards peripheral blood flow to support increased radiant heat dissipation through the skin. As a result, the gastrointestinal tract (GIT) integrity is compromised (Lambert, 2009; Pearce et al., 2013c). Decreased blood flow to the GIT reduces nutrient and oxygen delivery causing hypoxia and incomplete barrier function of the tight junction in the epithelial lining. Decreased oxygen delivery can result in hypoxia in cells that leads to damage and apoptosis through acidosis, depletion of ATP, and altered on pump activity (Lambert, 2009). As a result, heat stress impairs GIT integrity leading to a "leaky gut" allowing the permeation of endotoxin and proinflammatory cytokines from intestinal bacteria into the blood stream (Hall et al., 2001; Yan et al., 2006). Endotoxin mediates its effects in tissues by binding to the Toll-like 4 receptor (TLR4) (Frost et al., 2004). In skeletal muscle, TLR4 activation results in reduced fatty acid metabolism, and decreased metabolic flexibility (Frisard et al., 2010). Endotoxin induced inflammation, similar to heat stress, causes a shift in post-absorptive fuel selection by decreasing fatty acid oxidation and promoting glycolytic metabolism (O'Neill, 2011; Baumgard and Rhoads, 2013a). Therefore, targeting GIT health has potential to alleviate the negative consequences to performance and composition seen in heat stressed animals.

The impact of heat stress on carcass composition, meat quality, and safety

The shift in nutrient absorption and nutrient partitioning due to heat stress increases muscle degradation and alters carcass composition resulting in reduced carcass prices (Baumgard and Rhoads, 2013b). Animals raised in heat stress conditions have reduced muscle mass and increase

adipose tissue as seen in pigs (Close et al., 1971; Verstegen et al., 1978; Heath, 1983; Collin et al., 2001), poultry (Geraert et al., 1996; Ryder et al., 2004) and rodents (Katsumata et al., 1990). Lactating animals and younger animals are more resistant to heat stress induced changes to carcass composition due to their different thermal neutral zone, while larger animals are heavily impacted (Christon, 1988; Collin et al., 2001). While animals reared in heat stress conditions have decreased feed intake, previous studies have shown that their altered metabolism and body composition cannot exclusively be attributed to this feed restriction (Wheelock et al., 2010; Pearce et al., 2013b). Animals raised in thermoneutral conditions on a feed restricted diet will deposit protein at the expense of lipid accretion so that the carcass becomes leaner (Le Dividich et al., 1980; Van Milgen and Noblet, 2003; Oresanya et al., 2008; Zhao et al., 2018). The opposing nutrient partitioning between heat stressed animals and feed restricted animals is indictive of altered metabolism in heat stressed pigs.

In addition to the alterations in carcass composition, heat stress prior to slaughter can have profound impacts on meat quality. Stress responses due to hyperthermia occur mainly through the autonomic nervous system mediated by catecholamines. While catecholamines facilitate the increase in respiratory rates and redistribution of blood flow, they also promote energy utilization from body reserves by increasing glycogenolysis in muscle and repressing energy storage (Minton, 1994; Gregory, 2010; Afsal et al., 2018). Catecholamines accelerate glycogenolysis in muscle by stimulating the β_2-receptors which activates glycogen phosphorylase and inhibits glycogen synthase. This causes activation of glycogenolysis and prevents glycogenesis (Roach, 1990; Franch et al., 1999). Acute heat stress immediately before slaughter leads to pale soft and exudative (PSE) meat due to an accelerated muscle glycogenolysis, accumulation of protons, and a rapid pH decline early post-mortem (Owens et al., 2009; Matarneh et al., 2017). This is most commonly

observed in swine and poultry (Santos et al., 1997; Adzitey and Nurul, 2011; Freitas et al., 2017), however, can also become a problem in cattle (Kim et al., 2014; Warner et al., 2014). Chronic heat stress results in dry firm and dark (DFD) meat due to a depleted muscle glycogen reserve resulting in a slow pH decline and a high final pH with high water holding capacity (Gregory, 2010; Adzitey and Nurul, 2011). While this mainly occurs in ruminants (Mitlohner et al., 2002; Kadim et al., 2008), it is also observed in swine (D'Souza et al., 1998). Additionally, the risk of diminished safety increases in meat from heat stress animals because of reduced shelf life due to altered lipid and protein oxidation as well as bacterial growth and shedding (Mujahid et al., 2007; Wang et al., 2009). Heat stressed animals exhibit great bacterial shedding and increased risk of carcass contamination which has been observed in pigs (Moro et al., 2000), poultry (Skarp et al., 2016), and cattle (Renter et al., 2008). Summer months have a greater prevalence of foodborne illness outbreaks (Self et al., 2017); however, the underlying mechanism have not been fully elucidated as bacterial shedding and an increased presence of bacteria during summer is confounded with handling and transport practices. Nevertheless, it is likely that the impaired gastrointestinal function observed during heat stress and meat contamination are related (Lambert, 2009; Gabler and Pearce, 2015). The impact of heat stress on meat quality and food safety poses a threat to health and profitability.

Metabolic modifications due to heat stress

Protein

During heat stress, protein deposition is reduced through downregulation of protein synthesis at the transcriptional level (Jacob, 1995). Heat stress leads to protein denaturation, which causes protein to aggregate in the nucleus altering many molecular and cellular functions like DNA synthesis, replication and repair, heat stress represses RNA content, proteolytic rates, muscle

protein turnover (Streffer, 1988; Higashikubo et al., 1993; Temim et al., 2000; Hahn, 2012). Duration of heat stress influences protein metabolism. Acute heat stress increases protein catabolism, and reduces protein synthesis and nitrogen retention (Tabiri et al., 2000). Chronic heat stress decreases protein synthesis and protein deposition (Geraert et al., 1996; Temim et al., 2000).

Lipid

Despite reductions in feed intake due to heat stress, several studies have demonstrated decreases in fat oxidation and reductions in plasma non-esterified fatty-acid (NEFA) concentrations in rodents (Sanders et al., 2009), pigs (Pearce et al., 2013b), sheep (Sano et al., 1983), and cattle (Ronchi et al., 1999; Shwartz et al., 2009). Lipolytic enzyme activities are downregulated in poultry and swine which may help to alleviate heat generation (Geraert et al., 1996) despite heat stress increases in lipolytic agents; epinephrine, glucagon, and cortisol (Febbraio, 2001). However, lipoprotein lipase activity in adipose tissue is increased which may suggest a greater storage capacity for triglycerides in heat stressed animals (Sanders et al., 2009). During feed restriction, animals use free fatty acids (FFA) mobilized from adipose tissue to spare glucose for skeletal muscle deposition (Baumgard and Rhoads, 2013a). Feed restricted animals increase FFA concentration and fatty acid oxidation. However, in heat stress conditions, regardless of decreased feed intake, animals have been shown to have decreased FFA concentrations and fatty acid oxidation compared with thermoneutral feed restricted animals (Sano et al., 1983; Elsasser et al., 2009; Shwartz et al., 2009; Pearce et al., 2013b; Zhao et al., 2018). Despite a low plane of nutrition in heat stressed animals, they are unable to efficiently utilize FFA.

Carbohydrates

Heat stress increases hepatic glucose output as a result of increased glycogenolysis (Febbraio, 2001) and increased gluconeogenesis (Collins et al., 1980). Dietary carbohydrate

consumption prevents hepatic glucose production under normal conditions, however, in heat stress conditions exogenous sugars do not impede glucose output by the liver (Angus et al., 2001). This indicates that during heat stress, animals have an increased reliance on glucose as their fuel substrate over lipids (Rhoads et al., 2013). Hepatic pyruvate carboxylase is the rate-limiting enzyme that regulates alanine and lactate entry into gluconeogenesis. During heat stress the expression of pyruvate carboxylase and the input of lactate into the gluconeogenesis pathway is increased (Collins et al., 1980; Rhoads et al., 2009; O'brien et al., 2010; Wheelock et al., 2010). Heat stressed animals also exhibit increased plasma lactate levels (Streffer, 1988) and most lactate is taken up by extrahepatic tissues to use for energy (Baumgard and Rhoads, 2012) perhaps as a mechanism to spare glucose for tissues who utilize it as their obligate fuel source.

Insulin

Although heat stressed animals experience decreased feed intake, which is traditionally observed with a reduction in insulin, and that heat stress is a catabolic condition, an increase in basal insulin levels is observed in multiple species (Torlińska et al., 1987; Itoh et al., 1998; Wheelock et al., 2010; Pearce et al., 2013b; Sanz Fernandez et al., 2015). Previous studies have demonstrated that in addition to increased circulating insulin, insulin sensitivity is also increased (Rhoads et al., 2009; Sanz Fernandez et al., 2015). This increase in insulin is a result of increased pancreas secretion and not reduced circulating insulin removal (Baumgard and Rhoads, 2012). This increase in circulating insulin results in decreased blood glucose levels, despite increases in hepatic glucose output. In thermoneutral conditions, increased insulin concentrations shifts metabolism to favor glucose utilization and decreases fat oxidation because of insulins antilipolytic activity which reduces adipose tissue export of NEFA and increases cellular glucose uptake (Carpentier et al., 2005). Therefore, during heat stress, animals have a limited ability to mobilize

fat and are thus less able to support fuel substrate switches and creates metabolic inflexibility (Zhao et al., 2018).

Metabolic flexibility during heat stress

Skeletal muscle constitutes a large portion of body mass and can influence whole body metabolism through its fuel substrate usage. Metabolic flexibility describes the ability to switch between fuel substrates to adapt according to changes in activity, prevailing conditions, or energy demands (Goodpaster and Sparks, 2017). Substrate metabolism and energy utilization are synchronized processes and specific tissues have differing demands and fuel substrate preferences. Skeletal muscle tissue is responsible for the majority of glucose disposal and increases glucose utilization during heat stress. Fuel substrate preference switches from mostly lipid to mostly carbohydrate in during exercise heat stress in humans, and environmental heat stress in pigs and sheep (Sano et al., 1983; Febbraio et al., 1994; Hargreaves et al., 1996; Jentjens et al., 2002; Zhao et al., 2018) due to changes in blood flow away from fat stores while increasing flow from liver to muscle. In a previous study, heat stress reduced metabolic flexibility further evidencing that skeletal muscle may not use FFA as its primary fuel source during heat stress (Zhao et al., 2018). This indicates that under stress condition, muscle is less able to adapt causing negative consequences to muscle growth and function, which may be due to alterations in gut function.

Increased reactive oxygen species during heat stress

Reduced oxidative glucose capacity observed in skeletal muscle during heat stress may be due to mitochondrial dysfunction. Mitochondria are the principle site of O_2 consumption and are also primary source of reactive oxygen species (ROS) that are produced as a byproduct of respiration (Cadenas and Davies, 2000; Turrens, 2003). The production of ROS occurs during the electron transport chain in the redox center of the four enzyme complexes by reducing oxygen into

superoxide anion (Barja, 1999; Genova et al., 2003). Accumulation of ROS like superoxide occurs when production exceeds the cells capacity to detoxify and can lead to damage to proteins, DNA and lipids (Halliwell, 1990; Apel and Hirt, 2004). Heat stress has been shown to increase ROS production leading to oxidative stress and damage in pigs (Montilla et al., 2014; Liu et al., 2015a), dairy cattle (Bernabucci et al., 2002), sheep (Chauhan et al., 2014). Damage to mitochondria can be especially detrimental because they are the main source of energy production within most cells so that damage can weaken the capacity to adapt to increased energy demands due to stressors (Hubbard, 1989). This may be a result of heat stress decreasing protein expression, thermal deactivation of antioxidant enzymes, or regulatory proteins. Superoxide and ROS production can be mitigated by uncoupling the electron transport chain though uncoupling proteins or by increasing non-enzymatic antioxidants and antioxidant enzymes (Birben et al., 2012). Heat stress can alter the principal mitochondrial antioxidant pathway by inactivating superoxide dismutase activity (Yang and Lin, 2002; Belhadj Slimen et al., 2014), and can reduce the expression of uncoupling proteins (Mujahid et al., 2006). Because uncoupling proteins can help to control the amount of superoxide in the cell, their downregulation due to heat stress can lead to their accumulation, however, this may be a mechanism to reduce heat production since uncoupling proteins are responsible for non-shivering thermogenesis (Brand and Esteves, 2005). Uncoupling protein-3 (UCP3) is the main uncoupling protein found in skeletal muscle, and aside from thermogenesis and preventing ROS production, also functions to regulate the mitochondrial fatty acid oxidation (Cioffi et al., 2009). Without UCP3 mice have a lower ability to oxidize fat which may contribute to increased accumulation and storage of fatty acids. There is evidence that UCP3 may participate as a fatty acid anion carrier by translocating fatty acids out of the mitochondrial matrix (Goglia and Skulachev, 2003). Furthermore, leptin has been shown to increase the

expression of UCP3 in muscle (Henry et al., 2011). Since heat stress increases leptin (Morera et al., 2012), it is curious that UCP3 should be downregulated, however the necessity to conserve heat production may surpass the relationship between the two.

Methods to alleviate heat stress

Many management strategies have been implemented to alleviate the negative symptoms of heat stress in pig production, though few are effective and economically practical. Fans and evaporative cooling systems have been used to decrease ambient temperature, while floor cooling, drip cooling, and snout cooling have been used to increase animal heat loss (McGlone et al., 1988; Silva et al., 2006). Other strategies such as genetic selection for heat stress tolerance can be used to improve resistance to heat stress (Gourdine et al., 2006). However, these management strategies can be costly to producers, and even with these abatement interventions, the annual economic losses to the US swine industry during summer months were estimated to be $900 million (Pollman, 2010). Nutritional interventions could be implemented to mitigate heat stress, by the use of low increment diets or high-density diets that are correctly balanced (Renaudeau et al., 2008). Nutritional interventions are of particular interest due to the behavioral adaption of reduced feed intake during heat stress which reduces core body temperature, however, leads to negative consequences in performance and altered post absorptive nutrient metabolism. Therefore, the use of functional feed additives may provide an effective strategy to improve animal performance and gut functionality without increasing core body temperature.

Lipoic acid

Lipoic acid functions as a cofactor for mitochondrial enzymes that perform oxidative decarboxylation, can scavenge reactive oxygen species and nitrogen species, as well as improve cellular glucose uptake (Reed, 1974; Packer et al., 1995; Diesel et al., 2007). Lipoic acid mimics

insulin and can enhance cellular glucose uptake during thermoneutral conditions as seen in poultry and swine (Maddock et al., 2003; Hamano, 2006). Additionally, during heat stress administration of α-lipoic acid (ALA) to broiler chickens reduced oxidative stress (Imik et al., 2012). Furthermore, there is promising evidence that pretreatment with ALA may help to protect the intestinal epithelium from damage due to heat stress thus preserving its integrity (Varasteh et al., 2018).

Chromium

Chromium is an essential micronutrient that augments insulin through facilitating receptor binding of insulin to enhance its action on glucose, lipid, and protein metabolism (Mertz, 1993; Davis et al., 1997). A typical metabolic response of chromium supplementation is reduced plasma cortisol, especially during stress conditions, which may indicate chromium's alterations to insulin activity may be mediated by cortisol levels, since cortisol increases during stress and acts as an insulin antagonist (Chang and Mowat, 1992; Borgs and Mallard, 1998; Samanta et al., 2008). Chromium has been used in swine diets to improve growth performance and carcass characteristics, and has been shown to help to ameliorate the impact of heat stress when supplemented in summer months (Hung et al., 2014). Furthermore, supplementing pigs with chromium during cyclic heat stress conditions decreased rectal temperature and respiration rate (Liu et al., 2015b). Improvements to the heat stress response were also seen in dairy cow when supplemented with chromium by reducing weight loss, increasing milk production and reducing plasma NEFA concentrations (Soltan, 2010). Chickens had improved feed intake, weight gain, and carcass composition during heat stress when supplemented (Toghyani et al., 2012).

Antioxidants

Feed additives like vitamins and micro minerals that have antioxidant functions are promising supplements to reduce oxidative stress caused by heat stress (Renaudeau et al., 2012). Two groups of antioxidants exist; non-enzymatic and antioxidant enzymes. Non-enzymatic enzymes such as vitamin E, vitamin C, polyphenols, uric acid, and glutathione possess reducing abilities, while antioxidant enzymes such as catalase, superoxide dismutase, and glutathione peroxidase can neutralize free radicals or their metabolites (Cottrell et al., 2015). Giving antioxidant supplements during heat stress improved the physiological response in sheep (Chauhan et al., 2014), poultry (Maini et al., 2007), dairy cattle (Calamari et al., 2011), and pigs (Adenkola et al., 2009). In fact, at supra-nutritional levels of vitamin E was shown to alleviate the negative effects of heat stress by decreasing rectal temperature, respiration rates and increasing feed intake and improving oxidative balance in sheep (Chauhan et al., 2014). In pigs, vitamin C supplementation reduced rectal temperature (Adenkola et al., 2009), while zinc supplementation as well as vitamin E and selenium improved gut function (Fernandez et al., 2014; Pearce et al., 2015; Liu et al., 2016). Since animals with inadequate antioxidant defenses are more likely to be negatively affected by heat stress (Finch and Turner, 1996; Bottje and Carstens, 2009), providing sufficient antioxidant supplementation can aid in easing the negative effects of heat stress.

Artificial sweeteners

Supplementation with high-intensity artificial sweetener has been shown to improve gut function and feed intake, which are impaired with heat stress. Taste can influence feed consumption and is especially important when feeding behaviors are suppressed (Hellekant and Danilova, 1999). Supplementing with artificial sweeteners enhances chewing behavior in growing pigs, at the expense of exploratory behavior (Day et al., 1996). This may help to enhance the behavioral

adaption of decreased locomotion observed during heat stress. Despite reports of the artificial sweetener saccharin not being able to stimulate the cranial nerve and pigs having a difficult time tasting saccharin (Hellekant and Danilova, 1999), supplementation increased average daily gain and feed intake of in piglets (Lei et al., 2017) which are depressed during heat stress.

Artificial sweeteners improve gut integrity by working though taste receptors in the intestinal epithelium. These taste receptors can sense sugars and artificial sweeteners which increases the secretion of the gut hormone glucagon-like peptide (GLP)-2 and upregulates expression of the Na+/glucose co-transporter 1 (SGLT-1) through stimulating the G-coupled receptor (Drucker, 1996). Glucagon-like peptide-2 improves gut function and is a potent gastroprotective agent that increases blood flow (Stephens et al., 2006), stimulates mucosal growth (Moran et al., 2010; Vegge et al., 2013), reduces the inflammatory response (Cani et al., 2009), prevents cell death during stress (Burrin et al., 2007), and helps to maintain glucose homeostasis (Bahrami et al., 2010; Shi et al., 2013). A subset of enteroendocrine cells expressing these taste receptors excrete GLP-2, which contributes to the upregulation of SGLT-1. This upregulation of SGLT-1 by supplementing artificial sweeteners enhances the capacity of the gut to absorb dietary sugar, electrolytes, and water. Increased SGLT-1 is associated with an improved gut morphology via increases villus and tight junction strength in pigs and ruminants (Moran et al., 2010; Moran et al., 2014). This improved morphology and upregulation of SGLT1 from including artificial sweeteners in the diet provides protection against disease challenges such as *e.coli* and *cryptosporidium parvum* (Connor et al., 2017; Moran et al., 2019) by reducing morbidity and improving gut absorption. Therefore, this improved intestinal health and nutrient absorption can be targeted as a potential strategy to decrease the negative impacts of heat stress.

Capsicum

In addition to artificial sweeteners, Capsicum oleoresin has been shown to improve gut function. This feed additive is derived from hot peppers with the principle component, Capsaicin, being the major pungent hydrophobic alkaloid of the pepper (Cichewicz and Thorpe, 1996). The activity of capsaicin varies depending on the site of activation. The biological effects of capsaicin are carried out by "capsaicin-sensitive" sensory nerve (CPSN) fibers (Buck, 1986). Capsaicin binds to the transient receptor potential cation channel subfamily V member 1 (TrpV1) located on sensory nerve endings which causes the channel to open and the nerve endings depolarizes causing a burning sensation (Caterina et al., 1997). Along with capsaicin, noxious heat and acidic pH are able to activate the TRPV1 receptor, eliciting a response (Hinman et al., 2006). Repeated exposure to capsaicin causes a desensitization of the receptor and neurons become irresponsive to noxious stimuli (Liu and Simon, 1996; Koplas et al., 1997; Caterina et al., 1999; Yao and Qin, 2009). Stimulation of CPSN fibers enhances blood flow to the area (Holzer, 1988). A dense plexus of CPSN is found in the gastric submucosal blood vessels and play an important role in the regulation of blood flow to the gastric mucosa (Sharkey et al., 1984; Sternini et al., 1987; Grhkn and Dockray, 1988). Increased blood flow provides the gastric mucosa with a protective mechanism against noxious stimuli in the stomach (Yonei et al., 1990). Because of its vasodilatation effects, capsaicin can be used against heat stress to induce smooth muscle vasodilation (Chen et al., 1992). During heat stress, blood flow is diverted to the periphery, supplementing with capsaicin may help to maintain blood flow to the gut, therefore protecting against compromised integrity of the GIT.

When ingested, capsaicin binds to the TrpV1 receptor and is passively absorbed in the stomach and upper portion of the small intestine with greater than 80% efficiency (Kawada et al., 1984; Iwai et al., 2003). When in circulation, capsaicin is transported to the adrenal gland by

albumin and stimulates catecholamine release (Kawada et al., 1988). Previous studies have found that capsaicin stimulated catecholamine release improves lipolysis in adipocytes, liver glycogenolysis in the liver, and substrate oxidation in muscle tissue (Kawada et al., 1984; Watanabe et al., 1994; Reinbach, 2008) which may help relieve the alterations to substrate metabolism and utilization caused by heat stress.

Capsicum is able to aid in digestion and protects against bacterial infection in the gut. Previous studies have shown capsicum is capable of stimulating digestive enzymes and bile secretion (Platel and Srinivasan, 2000) and increasing fluid intake (Curtis and Stricker, 1997). The antimicrobial properties of capsaicin have been investigated as a natural alternative to antibiotic use in livestock (Meunier et al., 2007). Capsaicin effects the resistance to *Salmonella enteritidis* infection through altering pH and histological changes in chickens and has been shown to activate lipid metabolism networks in the intestinal intraepithelial lymphocytes (Kim et al., 2010). Capsicum oleoresin has anti-inflammatory effects on lymphocytes in the intestinal mucosa by modulating transcription of inflammatory genes (Kim et al., 2010; Nevius et al., 2012). When fed to weaned piglets, capsicum oleoresin decreased the incidence of diarrhea and decreased the severity of immune response when challenged with *e. coli* (Liu et al., 2013b), and improved growth performance and immune responses when challenged with porcine reproductive and respiratory syndrome virus (Liu et al., 2013a). The antimicrobial properties of capsicum along with its ability to increase blood flow and alter metabolism make it a promising product to alleviate heat stress. While nutritional interventions may not ameliorate all the negative consequences of heat stress, they are an economic solution to improve the health and performance of the animal during a time when losses are great.

Conclusion

In summary, muscle growth is a complex process that is impacted by both external environment and nutrition. Altering diet composition can lead to improved efficiency or have detrimental consequences. Understanding the impacts of nutrition in early life and alleviating environmental stressors during growth can provide valuable information on optimizing production.

References

Adebowale, T., J. Shunshun, and K. Yao. 2019. The effect of dietary high energy density and carbohydrate energy ratio on digestive enzymes activity, nutrient digestibility, amino acid utilization and intestinal morphology of weaned piglets. Journal of Animal Physiology and Animal Nutrition 103(5):1492-1502. doi: 10.1111/jpn.13123

Adenkola, A., J. Ayo, and A. Sackey. 2009. Ascorbic acid-induced modulation of rectal temperature fluctuations in pigs during the harmattan season. Journal of Thermal Biology 34(3):152-154. doi: 10.1016/j.jtherbio.2009.01.001

Adzitey, F., and H. Nurul. 2011. Pale soft exudative (PSE) and dark firm dry (DFD) meats: causes and measures to reduce these incidences-a mini review. International Food Research Journal 18(1)

Afsal, A., V. Sejian, M. Bagath, G. Krishnan, C. Devaraj, and R. Bhatta. 2018. Heat stress and livestock adaptation: neuro-endocrine regulation. doi: 10.31021/ijvam.20181108.

Alexander, L. S., B. S. Seabolt, R. P. Rhoads, and C. H. Stahl. 2012. Neonatal phosphate nutrition alters in vivo and in vitro satellite cell activity in pigs. Nutrients 4(6):436-448. doi: 10.3390/nu4060436

Allen, R. E., R. A. Merkel, and R. B. Young. 1979. Cellular aspect of muscle growth: myogenic cell proliferation. Journal of animal science 49(1):115-127.

Allen, R. E., S. M. Sheehan, R. G. Taylor, T. L. Kendall, and G. M. Rice. 1995. Hepatocyte growth factor activates quiescent skeletal muscle satellite cells in vitro. Journal of cellular physiology 165(2):307-312. doi: 10.1002/jcp.1041650211

Anderson, J. J., S. C. Garner, and P. J. Klemmer. 2016. Diet, nutrients, and bone health. CRC Press.

Angus, D. J., M. A. Febbraio, D. Lasini, and M. Hargreaves. 2001. Effect of carbohydrate ingestion on glucose kinetics during exercise in the heat. Journal of applied physiology 90(2):601-605. doi: 10.1152/jappl.2001.90.2.601

Apel, K., and H. Hirt. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. 55:373-399. doi: 10.1146/annurev.arplant.55.031903.141701

Aschenbach, J. R., K. Steglich, G. Gäbel, and K. U. Honscha. 2009. Expression of mRNA for glucose transport proteins in jejunum, liver, kidney and skeletal muscle of pigs. Journal of Physiology and Biochemistry 65(3):251-266. doi: 10.1007/BF03180578

Bach, A. C., and V. K. Babayan. 1982. Medium-chain triglycerides: an update. Am J Clin Nutr 36(5):950-962. doi: 10.1093/ajcn/36.5.950

Bahrami, J., C. Longuet, L. L. Baggio, K. Li, and D. J. Drucker. 2010. Glucagon-like peptide-2 receptor modulates islet adaptation to metabolic stress in the ob/ob mouse. Gastroenterology 139(3):857-868.

Bai, L., R. Liang, Y. Yang, X. Hou, Z. Wang, S. Zhu, C. Wang, Z. Tang, and K. Li. 2015. Microrna-21 regulates pi3k/akt/mtor signaling by targeting tgfβi during skeletal muscle development in pigs. PloS one 10(5)doi: 10.1371/journal.pone.0119396

Barja, G. 1999. Mitochondrial oxygen radical generation and leak: sites of production in states 4 and 3, organ specificity, and relation to aging and longevity. Journal of bioenergetics and biomembranes 31(4):347-366. doi: 10.1023/A:1005427919188

Barker, D. J. P. 2007. The origins of the developmental origins theory. Journal of Internal Medicine 261(5):412-417. doi: 10.1111/j.1365-2796.2007.01809.x

Bartel, D. P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. cell 116(2):281-297.

Baumgard, L., and R. Rhoads. 2012. Ruminant Nutrition Symposium: ruminant production and metabolic responses to heat stress. Journal of Animal Science 90(6):1855-1865. doi: 10.2527/jas.2011-4675

Baumgard, L. H., and R. P. Rhoads. 2013a. Effects of heat stress on postabsorptive metabolism and energetics. Annu. Rev. Anim. Biosci. 1(1):311-337.

Baumgard, L. H., and R. P. Rhoads. 2013b. Effects of heat stress on postabsorptive metabolism and energetics. Annu. Rev. Anim. Biosci. doi: 10.1146/annurev-animal-031412-103644

Behm-Ansmant, I., J. Rehwinkel, T. Doerks, A. Stark, P. Bork, and E. Izaurralde. 2006. mRNA degradation by miRNAs and GW182 requires both CCR4: NOT deadenylase and DCP1: DCP2 decapping complexes. Genes & development 20(14):1885-1898.

Belhadj Slimen, I., T. Najar, A. Ghram, H. Dabbebi, M. Ben Mrad, and M. Abdrabbah. 2014. Reactive oxygen species, heat stress and oxidative-induced mitochondrial damage. A review. International journal of hyperthermia 30(7):513-523. doi: 10.3109/02656736.2014.971446

Bell, M. L., M. Buvoli, and L. A. Leinwand. 2010. Uncoupling of expression of an intronic microRNA and its myosin host gene by exon skipping. Molecular and cellular biology 30(8):1937-1945. doi: 10.1128/MCB.01370-09

Berman, A. 2006. Extending the potential of evaporative cooling for heat-stress relief. Journal of dairy science 89(10):3817-3825. doi: 10.3168/jds.S0022-0302(06)72423-7

Bernabucci, U., N. Lacetera, L. H. Baumgard, R. P. Rhoads, B. Ronchi, and A. Nardone. 2010. Metabolic and hormonal acclimation to heat stress in domesticated ruminants. Animal 4(7):1167-1183. doi: 10.1017/S175173111000090X

Bernabucci, U., B. Ronchi, N. Lacetera, and A. Nardone. 2002. Markers of oxidative status in plasma and erythrocytes of transition dairy cows during hot season. Journal of dairy science 85(9):2173-2179. doi: 10.3168/jds.S00220302(02)74296-3

Bernstein, E., S. Y. Kim, M. A. Carmell, E. P. Murchison, H. Alcorn, M. Z. Li, A. A. Mills, S. J. Elledge, K. V. Anderson, and G. J. Hannon. 2003. Dicer is essential for mouse development. Nature genetics 35(3):215.

Binder, H. J., and A. Reuben. 2005. Nutrient digestion and absorption, Medical physiology: a cellular and molecular approach. Updated ed. Elsevier Saunders, Philadelphia. p. 947-974.

Birben, E., U. M. Sahiner, C. Sackesen, S. Erzurum, and O. Kalayci. 2012. Oxidative Stress and Antioxidant Defense. World Allergy Organization Journal 5(1):9-19. doi: 10.1097/WOX.0b013e3182439613

Bishop-Williams, K. E., O. Berke, D. L. Pearl, K. Hand, and D. F. Kelton. 2015. Heat stress related dairy cow mortality during heat waves and control periods in rural Southern Ontario from 2010–2012. BMC veterinary research 11(1):291.

Bjornson, C. R., T. H. Cheung, L. Liu, P. V. Tripathi, K. M. Steeper, and T. A. Rando. 2012. Notch signaling is necessary to maintain quiescence in adult muscle stem cells. Stem Cells 30(2):232-242. doi: 10.1002/stem.773

Black, J., B. Mullan, M. Lorschy, and L. Giles. 1993. Lactation in the sow during heat stress. Livestock production science 35(1-2):153-170. doi: 10.1016/0301-6226(93)90188-N

Borgs, P., and B. Mallard. 1998. Immune-endocrine interactions in agricultural species: chromium and its effect on health and performance. Domestic Animal Endocrinology 15(5):431-438. doi: 10.1016/S0739-7240(98) 00018-6

Bottje, W., and G. Carstens. 2009. Association of mitochondrial function and feed efficiency in poultry and livestock species. Journal of animal science 87(suppl_14):E48-E63. doi: 10.2527/jas.2008-1379

Brand, M. D., and T. C. Esteves. 2005. Physiological functions of the mitochondrial uncoupling proteins UCP2 and UCP3. Cell metabolism 2(2):85-93.

Bringhurst, F., and B. Leder. 2006. Regulation of calcium and phosphate homeostasis. Endocrinology. Fifth ed. Philadelphia: WB Saunders Co:805-843.

Bronner, F., D. Pansu, and W. Stein. 1986. An analysis of intestinal calcium transport across the rat intestine. American Journal of Physiology-Gastrointestinal and Liver Physiology 250(5):G561-G569. doi: 10.1152/ajpgi.1986.250.5.G561

Buck, S. H. 1986. The neuropharmacology of capsaicin: review of some recent observations. Pharmacol. Rev. 38:179-226.

Buckingham, M. 2007. Skeletal muscle progenitor cells and the role of Pax genes. Comptes rendus biologies 330(6-7):530-533.

Burrin, D. G., B. Stoll, X. Guan, L. Cui, X. Chang, and D. Hadsell. 2007. GLP-2 rapidly activates divergent intracellular signaling pathways involved in intestinal cell survival and proliferation in neonatal piglets. American Journal of Physiology-Endocrinology and Metabolism 292(1):E281-E291. doi: 10.1146/annurev-animal-031412-103644

Cadenas, E., and K. J. Davies. 2000. Mitochondrial free radical generation, oxidative stress, and aging. Free Radical Biology and Medicine 29(3-4):222-230. doi: 10.1016/s0891-5849(00)00317-8

Calamari, L., F. Petrera, F. Abeni, and G. Bertin. 2011. Metabolic and hematological profiles in heat stressed lactating dairy cows fed diets supplemented with different selenium sources and doses. Livestock Science 142(1-3):128-137. doi: 10.1016/j.livsci.2011.07.005

Callis, T. E., K. Pandya, H. Y. Seok, R.-H. Tang, M. Tatsuguchi, Z.-P. Huang, J.-F. Chen, Z. Deng, B. Gunn, and J. Shumate. 2009. MicroRNA-208a is a regulator of cardiac hypertrophy and conduction in mice. The Journal of clinical investigation 119(9):2772-2786. doi: 10.1172/JCI36154

Cani, P. D., S. Possemiers, T. Van de Wiele, Y. Guiot, A. Everard, O. Rottier, L. Geurts, D. Naslain, A. Neyrinck, and D. M. Lambert. 2009. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut 58(8):1091-1103.

Carey, M. C., D. M. Small, and C. M. Bliss. 1983. Lipid Digestion and Absorption. Annual Review of Physiology 45(1):651-677. doi: 10.1146/annurev.ph.45.030183.003251

Carpenter, D. A., F. P. O'Mara, and J. V. O'Doherty. 2004. The Effect of Dietary Crude Protein Concentration on Growth Performance, Carcass Composition and Nitrogen Excretion in Entire Grower-Finisher Pigs. Irish Journal of Agricultural and Food Research 43(2):227-236.

Carpentier, A. C., F. Frisch, D. Cyr, P. Généreux, B. W. Patterson, R. Giguère, and J.-P. Baillargeon. 2005. On the suppression of plasma nonesterified fatty acids by insulin during enhanced intravascular lipolysis in humans. American Journal of Physiology-Endocrinology and Metabolism 289(5):E849-E856. doi: 10.1152/ajpendo.00073.2005

Carr, T. R., L. E. Walters, and J. V. Whiteman. 1978. Carcass Composition Changes in Growing and Finishing Swine. Journal of Animal Science 47(3):615-621. doi: 10.2527/jas1978.473615x

Caterina, M. J., T. A. Rosen, M. Tominaga, A. J. Brake, and D. Julius. 1999. A capsaicin-receptor homologue with a high threshold for noxious heat. Nature 398(6726):436-441. doi: 10.1038/18906

Caterina, M. J., M. A. Schumacher, M. Tominaga, T. A. Rosen, J. D. Levine, and D. Julius. 1997. The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389(6653):816-824. doi: 10.1038/39807

Chang, X., and D. Mowat. 1992. Supplemental chromium for stressed and growing feeder calves. Journal of Animal Science 70(2):559-565. doi: 10.2527/1992.702559x

Chauhan, S. S., P. Celi, B. J. Leury, I. J. Clarke, and F. R. Dunshea. 2014. Dietary antioxidants at supranutritional doses improve oxidative status and reduce the negative effects of heat stress in sheep. Journal of Animal Science 92(8):3364-3374. doi: 10.2527/jas.2014-7714

Chen, H.-Y., A. J. Lewis, P. S. Miller, and J. T. Yen. 1999. The effect of excess protein on growth performance and protein metabolism of finishing barrows and gilts. Journal of Animal Science 77(12):3238-3247. doi: 10.2527/1999.77123238x

Chen, J.-F., E. M. Mandel, J. M. Thomson, Q. Wu, T. E. Callis, S. M. Hammond, F. L. Conlon, and D.-Z. Wang. 2006. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nature genetics 38(2):228.

Chen, R., D.-S. Li, and P. H. Guth. 1992. Role of calcitonin gene-related peptide in capsaicin-induced gastric submucosal arteriolar dilation. American Journal of Physiology-Heart and Circulatory Physiology 262(5):H1350-H1355. doi: 10.1152/ajpheart.1992.262.5.H1350

Cheung, T. H., N. L. Quach, G. W. Charville, L. Liu, L. Park, A. Edalati, B. Yoo, P. Hoang, and T. A. Rando. 2012. Maintenance of muscle stem-cell quiescence by microRNA-489. Nature 482(7386):524-528. doi: 10.1038/nature10834

Chiba, L. I. 1995. Effects of nutritional history on the subsequent and overall growth performance and carcass traits of pigs. Livestock Production Science 41(2):151-161. doi: 10.1016/0301-6226(94)00050-H

Chiba, L. I., D. L. Kuhlers, L. T. Frobish, S. B. Jungst, E. J. Huff-Lonergan, S. M. Lonergan, and K. A. Cummins. 2002. Effect of dietary restrictions on growth performance and carcass quality of pigs selected for lean growth efficiency. Livestock Production Science 74(1):93-102. doi: 10.1016/S0301-6226(01)00288-3

Christon, R. 1988. The effect of tropical ambient temperature on growth and metabolism in pigs. Journal of Animal Science 66(12):3112-3123. doi: 10.2527/jas1988.66123112x

Cichewicz, R. H., and P. A. Thorpe. 1996. The antimicrobial properties of chile peppers (Capsicum species) and their uses in Mayan medicine. Journal of ethnopharmacology 52(2):61-70. doi: 10.1016/0378-8741(96)01384-0

Cioffi, F., R. Senese, P. de Lange, F. Goglia, A. Lanni, and A. Lombardi. 2009. Uncoupling proteins: a complex journey to function discovery. Biofactors 35(5):417-428. doi: 10.1002/biof.54

Close, W., L. Mount, and I. Start. 1971. The influence of environmental temperature and plane of nutrition on heat losses from groups of growing pigs. Animal Science 13(2):285-294. doi: 10.1017/S00033 5610002972X

Collin, A., J. Van Milgen, S. Dubois, and J. Noblet. 2001. Effect of high temperature and feeding level on energy utilization in piglets. Journal of animal science 79(7):1849-1857. doi: 10.2527/2001.7971849x

Collins, F. G., F. A. Mitros, and J. L. Skibba. 1980. Effect of palmitate on hepatic biosynthetic functions at hyperthermic temperatures. Metabolism 29(6):524-531. doi: 10.1016/0026-0495(80)90077-3

Connor, E., E. Wall, D. Bravo, C. Evock-Clover, T. Elsasser, R. B. VI, M. Santín, B. Vinyard, S. Kahl, and M. Walker. 2017. Reducing gut effects from Cryptosporidium parvum infection in dairy calves through prophylactic glucagon-like peptide 2 therapy or feeding of an artificial sweetener. Journal of dairy science 100(4):3004-3018.

Cooper, R. N., S. Tajbakhsh, V. Mouly, G. Cossu, M. Buckingham, and G. S. Butler-Browne. 1999. In vivo satellite cell activation via Myf5 and MyoD in regenerating mouse skeletal muscle. J Cell Sci 112 (Pt 17):2895-2901.

Cordell, D., J.-O. Drangert, and S. White. 2009. The story of phosphorus: global food security and food for thought. Global environmental change 19(2):292-305. doi: 10.1016/j.gloenvcha.2008.10.009

Cottrell, J., F. Liu, A. Hung, K. DiGiacomo, S. Chauhan, B. Leury, J. Furness, P. Celi, and F. Dunshea. 2015. Nutritional strategies to alleviate heat stress in pigs. Animal Production Science 55(12):1391-1402. doi: 10.1071/AN15255

Cromwell, G. L., R. D. Coffey, H. J. Monegue, and J. H. Randolph. 1995. Efficacy of low-activity, microbial phytase in improving the bioavailability of phosphorus in corn-soybean meal diets for pigs. J Anim Sci 73(2):449-456. doi: 10.2527/1995.732449x

Cross, H., H. Debiec, and M. Peterlik. 1990. Mechanism and regulation of intestinal phosphate absorption. Miner Electrolyte Metab 16(2-3):115-124.

Curtis, K. S., and E. M. Stricker. 1997. Enhanced fluid intake by rats after capsaicin treatment. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 272(2):R704-R709. doi: 10.1152/ajpregu.1997.272.2.R704

Curtis, S. E. 1983. Environmental Management in Animal Agriculture. Iowa State University Press, Ames, Iowa.

Czech, B., and G. J. Hannon. 2011. Small RNA sorting: matchmaking for Argonautes. Nature Reviews Genetics 12(1):19-31. doi: 10.1038/nrg2916

D'Allaire, S., R. Drolet, and D. Brodeur. 1996. Sow mortality associated with high ambient temperatures. The Canadian Veterinary Journal 37(4):237.

D'Souza, D., B. Leury, F. Dunshea, and R. Warner. 1998. Effect of on-farm and pre-slaughter handling of pigs on meat quality. Australian Journal of Agricultural Research 49(6):1021-1025.

D'Souza, D. M., K. E. Trajcevski, D. Al-Sajee, D. C. Wang, M. Thomas, J. E. Anderson, and T. J. Hawke. 2015. Diet-induced obesity impairs muscle satellite cell activation and muscle repair through alterations in hepatocyte growth factor signaling. Physiological Reports 3(8):e12506. doi: 10.14814/phy2.12506

Davis, B. N., A. C. Hilyard, G. Lagna, and A. Hata. 2008. SMAD proteins control DROSHA-mediated microRNA maturation. Nature 454(7200):56-61. doi: 10.1038/nature07086

Davis, B. N., A. C. Hilyard, P. H. Nguyen, G. Lagna, and A. Hata. 2010. Smad proteins bind a conserved RNA sequence to promote microRNA maturation by Drosha. Molecular cell 39(3):373-384. doi: 10.1016/j.molcel.2010.07.011

Davis, C. M., A. C. Royer, and J. B. Vincent. 1997. Synthetic multinuclear chromium assembly activates insulin receptor kinase activity: functional model for low-molecular-weight chromium-binding substance. Inorganic chemistry 36(23):5316-5320. doi: 10.1021/ic970568h

Davis, T. A., D. G. Burrin, M. L. Fiorotto, and H. V. Nguyen. 1996. Protein synthesis in skeletal muscle and jejunum is more responsive to feeding in 7-than in 26-day-old pigs. American Journal of Physiology-Endocrinology and Metabolism 270(5):E802-E809. doi: 10.1152/ajpendo.1996.270.5.E802

Davis, T. A., M. L. Fiorotto, H. V. Nguyen, and P. J. Reeds. 1989. Protein turnover in skeletal muscle of suckling rats. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 257(5):R1141-R1146. doi: 10.1152/ajpregu.1989.257.5.R1141

Day, J., I. Kyriazakis, and A. Lawrence. 1996. An investigation into the causation of chewing behaviour in growing pigs: the role of exploration and feeding motivation. Applied Animal Behaviour Science 48(1-2):47-59. doi: 10.1016/0168-1591(95)01022-X

Delgado, C. 2005. Rising demand for meat and milk in developing countries: implications for grasslands-based livestock production. Grassland: a global resource:29-39.

DeLuca, H. F. 2008. Evolution of our understanding of vitamin D. Nutrition reviews 66(suppl_2):S73-S87. doi: 10.1111/j.1753-4887.2008.00105.x

den Dekker, E., J. G. Hoenderop, B. Nilius, and R. J. Bindels. 2003. The epithelial calcium channels, TRPV5 & TRPV6: from identification towards regulation. Cell calcium 33(5-6):497-507. doi: 10.1016/S0143-4160(03)00065-4

Devlin, T. M. 2006. Textbook of biochemistry: with clinical correlations. Wiley-Liss Hoboken, NJ.

Dey, B. K., J. Gagan, and A. Dutta. 2011. miR-206 and-486 induce myoblast differentiation by downregulating Pax7. Molecular and cellular biology 31(1):203-214.

Diesel, B., S. Kulhanek-Heinze, M. Höltje, B. Brandt, H.-D. Höltje, A. M. Vollmar, and A. K. Kiemer. 2007. α-Lipoic acid as a directly binding activator of the insulin receptor: protection from hepatocyte apoptosis. Biochemistry 46(8):2146-2155. doi: 10.1021/bi602547m

Doench, J. G., and P. A. Sharp. 2004. Specificity of microRNA target selection in translational repression. Genes & development 18(5):504-511.

Driver, J. P., G. M. Pesti, R. I. Bakalli, and H. M. Edwards. 2006. The Effect of Feeding Calcium- and Phosphorus-Deficient Diets to Broiler Chickens During the Starting and Growing-Finishing Phases on Carcass Quality. Poultry Science 85(11):1939-1946. doi: 10.1093/ps/85.11.1939

Drucker, D. 1996. Ehrlich P, Asa SL, Brubaker PL. Induction of intestinal epithelial proliferation by glucagon-like peptide 2:7911-7916. doi: 10.1073/pnas.93.15.7911

Drummond, M. J., E. L. Glynn, C. S. Fry, S. Dhanani, E. Volpi, and B. B. Rasmussen. 2009. Essential amino acids increase microRNA-499,-208b, and-23a and downregulate myostatin and myocyte enhancer factor 2C mRNA expression in human skeletal muscle. The Journal of nutrition 139(12):2279-2284. doi: 10.3945/jn.109.112797

Drummond, M. J., J. J. McCarthy, C. S. Fry, K. A. Esser, and B. B. Rasmussen. 2008. Aging differentially affects human skeletal muscle microRNA expression at rest and after an anabolic stimulus of resistance exercise and essential amino acids. American Journal of Physiology-Endocrinology and Metabolism 295(6):E1333-E1340. doi: 10.1152/ajpendo.90562.2008

Drummond, M. J., J. J. McCarthy, M. Sinha, H. M. Spratt, E. Volpi, K. A. Esser, and B. B. Rasmussen. 2011. Aging and microRNA expression in human skeletal muscle: a microarray and bioinformatics analysis. Physiological genomics 43(10):595-603. doi: 10.1152/physiolgenomics.00148.2010

Elia, L., R. Contu, M. Quintavalle, F. Varrone, C. Chimenti, M. A. Russo, V. Cimino, L. De Marinis, A. Frustaci, and D. Catalucci. 2009. Reciprocal regulation of microRNA-1 and IGF-1 signal transduction cascade in cardiac and skeletal muscle in physiological and pathological conditions. Circulation 120(23):2377. doi: 10.1161/CIRCULATIONAHA.109.879429

Elsasser, T., R. Rhoads, S. Kahl, R. Collier, L. Baumgard, C. Li, and T. Caperna. 2009. Heat stress augments plasma tyrosine-nitrated proteins and lactate-to-pyruvate ratio after repeated endotoxin (LPS) challenge in steers. J. Anim. Sci 87(9):47-59.

Endo, M. 1977. Calcium release from the sarcoplasmic reticulum. Physiological reviews 57(1):71-108. doi: 10.1152/physrev.1977.57.1.71

Eulalio, A., E. Huntzinger, and E. Izaurralde. 2008. GW182 interaction with Argonaute is essential for miRNA-mediated translational repression and mRNA decay. Nature structural & molecular biology 15(4):346.

Fang, L. H., Y. H. Jin, S. H. Do, J. S. Hong, B. O. Kim, T. H. Han, and Y. Y. Kim. 2019. Effects of dietary energy and crude protein levels on growth performance, blood profiles, and nutrient digestibility in weaning pigs. Asian-Australas J Anim Sci 32(4):556-563. doi: 10.5713/ajas.18.0294

Febbraio, M., R. Snow, C. Stathis, M. Hargreaves, and M. Carey. 1994. Effect of heat stress on muscle energy metabolism during exercise. Journal of Applied Physiology 77(6):2827-2831. doi: 10.1152/jappl.1994.77.6.2827

Febbraio, M. A. 2001. Alterations in energy metabolism during exercise and heat stress. Sports medicine 31(1):47-59.

Feng, Y., J. H. Cao, X. Y. Li, and S. H. Zhao. 2011a. Inhibition of miR-214 expression represses proliferation and differentiation of C2C12 myoblasts. Cell biochemistry and function 29(5):378-383. doi: 10.1002/cbf.1760

Feng, Y., X. Zhang, Q. Song, T. Li, and Y. Zeng. 2011b. Drosha processing controls the specificity and efficiency of global microRNA expression. Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms 1809(11-12):700-707. doi: 10.1016/j.bbagrm.2011.05.015

Fernandez, M. S., S. Pearce, N. Gabler, J. Patience, M. Wilson, M. Socha, J. Torrison, R. Rhoads, and L. Baumgard. 2014. Effects of supplemental zinc amino acid complex on gut integrity in heat-stressed growing pigs. Animal 8(1):43-50. doi: 10.1017/S1751731113001961

Finch, J., and R. Turner. 1996. Effects of selenium and vitamin E on the immune responses of domestic animals. Research in Veterinary Science 60(2):97-106. doi: 10.1016/S0034-5288(96)90001-6

Fiorotto, M. L., T. A. Davis, H. A. Sosa, C. Villegas-Montoya, I. Estrada, and R. Fleischmann. 2014. Ribosome abundance regulates the recovery of skeletal muscle protein mass upon recuperation from postnatal undernutrition in mice. The Journal of Physiology 592(23):5269-5286. doi: 10.1113/jphysiol.2014.279067

Franch, J., R. Aslesen, and J. Jensen. 1999. Regulation of glycogen synthesis in rat skeletal muscle after glycogen-depleting contractile activity: effects of adrenaline on glycogen

synthesis and activation of glycogen synthase and glycogen phosphorylase. Biochemical Journal 344(1):231-235. doi: 10.1042/bj3440231.
Freitas, A. S., L. M. Carvalho, A. L. Soares, M. E. d. S. Oliveira, M. S. Madruga, A. C. d. S. Neto, R. H. Carvalho, E. I. Ida, and M. Shimokomaki. 2017. Pale, soft and exudative (PSE) and dark, firm and dry (DFD) meat determination in broiler chicken raised under tropical climate management conditions. Int J Poult Sci 16:81-87.
Friedman, R. C., K. K.-H. Farh, C. B. Burge, and D. P. Bartel. 2009. Most mammalian mRNAs are conserved targets of microRNAs. Genome research 19(1):92-105. doi: 10.1016/j.cell.2004.12.035
Frisard, M. I., R. P. McMillan, and M.-J. of …. 2010. Toll-like receptor 4 modulates skeletal muscle substrate metabolism. American Journal of …
Frost, R. A., G. J. Nystrom, and C. H. Lang. 2004. Lipopolysaccharide stimulates nitric oxide synthase-2 expression in murine skeletal muscle and C2C12 myoblasts via Toll-like receptor-4 and c-Jun NH2-terminal kinase pathways. American Journal of Physiology-Cell Physiology 287(6):C1605-C1615. doi: 10.1152/ajpcell.00010.2004
Fukada, S., A. Uezumi, M. Ikemoto, S. Masuda, M. Segawa, N. Tanimura, H. Yamamoto, Y. Miyagoe-Suzuki, and S. Takeda. 2007. Molecular signature of quiescent satellite cells in adult skeletal muscle. Stem Cells 25(10):2448-2459. doi: 10.1634/stemcells.2007-0019
Fulco, M., and V. Sartorelli. 2008. Comparing and contrasting the roles of AMPK and SIRT1 in metabolic tissues. Cell Cycle 7(23):3669-3679. doi: 10.4161/cc.7.23.7164
Fulco, M., R. L. Schiltz, S. Iezzi, M. T. King, P. Zhao, Y. Kashiwaya, E. Hoffman, R. L. Veech, and V. Sartorelli. 2003. Sir2 regulates skeletal muscle differentiation as a potential sensor of the redox state. Mol Cell 12(1):51-62. doi: 10.1016/s1097-2765(03)00226-0
Gabler, N., and S. Pearce. 2015. The impact of heat stress on intestinal function and productivity in grow-finish pigs. Animal production science 55(12):1403-1410. doi: 10.1071/AN15280.
Ge, Y., Y. Sun, and J. Chen. 2011. IGF-II is regulated by microRNA-125b in skeletal myogenesis. Journal of Cell Biology 192(1):69-81. doi: 10.1083/jcb.201007165
Genova, M. L., M. M. Pich, A. Biondi, A. Bernacchia, A. Falasca, C. Bovina, G. Formiggini, G. P. Castelli, and G. Lenaz. 2003. Mitochondrial production of oxygen radical species and the role of Coenzyme Q as an antioxidant. Experimental biology and medicine 228(5):506-513. doi: 10.1177/15353702-0322805-14
Georges, M., A. Clop, F. Marcq, H. Takeda, D. Pirottin, S. Hiard, X. Tordoir, F. Caiment, F. Meish, and B. Bibe. 2006. Polymorphic Polymorphic MicroRNA–Target Interactions: A Novel Source of Phenotypic Variation. In: Cold Spring Harbor symposia on quantitative biology. p 343-350.
Geraert, P., J. Padilha, and S. Guillaumin. 1996. Metabolic and endocrine changes induced by chronic heatexposure in broiler chickens: growth performance, body composition and energy retention. British Journal of Nutrition 75(2):195-204. doi: 10.1017/BJN19960124
Goglia, F., and V. P. Skulachev. 2003. A function for novel uncoupling proteins: antioxidant defense of mitochondrial matrix by translocating fatty acid peroxides from the inner to the outer membrane leaflet. The FASEB journal 17(12):1585-1591. doi: 10.1096/fj.03-0159hyp
Goljanek-Whysall, K., D. Sweetman, and A. E. Muensterberg. 2012. microRNAs in skeletal muscle differentiation and disease. Clinical science 123(11):611-625. doi: 10.1042/CS20110634

González-Vega, J., Y. Liu, J. C. McCann, C. Walk, J. J. Loor, and H.-H. Stein. 2016. Requirement for digestible calcium by eleven-to twenty-five–kilogram pigs as determined by growth performance, bone ash concentration, calcium and phosphorus balances, and expression of genes involved in transport of calcium in intestinal and kidney cells. Journal of animal science 94(8):3321-3334. doi: 10.2527/jas.2016-0444

Goodpaster, B. H., and L. M. Sparks. 2017. Metabolic flexibility in health and disease. Cell metabolism 25(5):1027-1036. doi: 10.1016/j.cmet.2017.04.015

Gourdine, J., J. Bidanel, J. Noblet, and D. Renaudeau. 2006. Effects of breed and season on performance of lactating sows in a tropical humid climate. Journal of animal science 84(2):360-369.

Gregory, N. 2010. How climatic changes could affect meat quality. Food Research International 43(7):1866-1873. doi: 10.1016/j.foodres.2009.05.018.

Grhkn, T., and G. J. Dockray. 1988. Characterization of the peptidergic afferent innervation of the stomach in the rat, mouse and guinea-pig. Neuroscience 25(1):181-193. doi: 10.1016/0306-4522(88)90017-6

Hagiwara, N., M. Yeh, and A. Liu. 2007. Sox6 is required for normal fiber type differentiation of fetal skeletal muscle in mice. Developmental dynamics: an official publication of the American Association of Anatomists 236(8):2062-2076. doi: 10.1002/dvdy.21223

Hahn, G. 1999. Dynamic responses of cattle to thermal heat loads. Journal of animal science 77(suppl_2):10-20.

Hahn, G. M. 2012. Hyperthermia and cancer. Springer Science & Business Media.

Halevy, O., A. Geyra, M. Barak, Z. Uni, and D. Sklan. 2000. Early Posthatch Starvation Decreases Satellite Cell Proliferation and Skeletal Muscle Growth in Chicks. The Journal of Nutrition 130(4):858-864. doi: 10.1093/jn/130.4.858

Halevy, O., Y. Nadel, M. Barak, I. Rozenboim, and D. Sklan. 2003. Early Posthatch Feeding Stimulates Satellite Cell Proliferation and Skeletal Muscle Growth in Turkey Poults. The Journal of Nutrition 133(5):1376-1382. doi: 10.1093/jn/133.5.1376

Hall, D. M., G. R. Buettner, L. W. Oberley, L. Xu, R. D. Matthes, and C. V. Gisolfi. 2001. Mechanisms of circulatory and intestinal barrier dysfunction during whole body hyperthermia. American Journal of Physiology-Heart and Circulatory Physiology 280(2):H509-H521. doi: 10.1152/ajpheart.2001.280.2.H509

Halliwell, G. 1990. Oxygen radicals in biological systems: part B. Academic Press, San Diego (CA).

Hamano, Y. 2006. Effects of dietary lipoic acid on plasma lipid, in vivo insulin sensitivity, metabolic response to corticosterone and in vitro lipolysis in broiler chickens. British journal of nutrition 95(6):1094-1101. doi: 10.1079/BJN20061766

Hamilton, A. J., and D. C. Baulcombe. 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286(5441):950-952.

Hansard, S. L., and H. M. Crowder. 1957. The Physiological Behavior of Calcium in the Rat. The Journal of Nutrition 62(3):325-339. doi: 10.1093/jn/62.3.325

Hansen, B. C., and A. J. Lewis. 1993. Effects of dietary protein concentration (corn:soybean meal ratio) on the performance and carcass characteristics of growing boars, barrows, and gilts: mathematical descriptions. Journal of Animal Science 71(8):2122-2132. doi: 10.2527/1993.7182122x

Hansen, P. J. 2009. Effects of heat stress on mammalian reproduction. Philosophical Transactions of the Royal Society B: Biological Sciences 364(1534):3341-3350. doi: doi:10.1098/rstb.2009.0131

Haque, N., A. Ludri, S. Hossain, and M. Ashutosh. 2013. Impact on hematological parameters in young and adult Murrah buffaloes exposed to acute heat stress. Buffalo Bull 32(4):321-326.

Hargreaves, M., D. Angus, K. Howlett, N. M. Conus, and M. Febbraio. 1996. Effect of heat stress on glucose kinetics during exercise. Journal of applied physiology 81(4):1594-1597. doi: 10.1152/jappl.1996.81.4.1594.

Hausburg, M. A., J. D. Doles, S. L. Clement, A. B. Cadwallader, M. N. Hall, P. J. Blackshear, J. Lykke-Andersen, and B. B. Olwin. 2015. Post-transcriptional regulation of satellite cell quiescence by TTP-mediated mRNA decay. Elife 4:e03390. doi: 10.7554/eLife.03390

Heath, M. E. 1983. The effects of rearing-temperature on body composition in young pigs. Comparative Biochemistry and Physiology Part A: Physiology 76(2):363-366. doi: 10.1016/0300-9629(83)90338-9

Hellekant, G., and V. Danilova. 1999. Taste in domestic pig, Sus scrofa. Journal of Animal Physiology and Animal Nutrition 82(1):8-24. doi: 10.1046/j.1439-0396.1999.00206.x

Hennessy, D., and P. Williamson. 1984. Stress and summer infertility in pigs. Australian Veterinary Journal 61(7):212-215.

Henry, B. A., Z. B. Andrews, A. Rao, and I. J. Clarke. 2011. Central Leptin Activates Mitochondrial Function and Increases Heat Production in Skeletal Muscle. Endocrinology 152(7):2609-2618. doi: 10.1210/en.2011-0143

Higashikubo, R., R. White, and J. Roti. 1993. Flow cytometric BrdUrd-pulse-chase study of heat-induced cell-cycle progression delays. Cell proliferation 26(4):337-348. doi: 10.1111/j.1365-2184.1993.tb00329.x

Hinman, A., H.-h. Chuang, D. M. Bautista, and D. Julius. 2006. TRP channel activation by reversible covalent modification. Proceedings of the National Academy of Sciences 103(51):19564-19568. doi: 10.1073/pnas.0609598103

Hocquette, J. F., I. Ortigues-Marty, D. Pethick, P. Herpin, and X. Fernandez. 1998. Nutritional and hormonal regulation of energy metabolism in skeletal muscles of meat-producing animals. Livestock Production Science 56(2):115-143. doi: 10.1016/S0301-6226(98)00187-0

Hoenderop, J. G., B. Nilius, and R. J. Bindels. 2005. Calcium absorption across epithelia. Physiological reviews 85(1):373-422. doi: 10.1152/physrev.00003.2004

Holloszy, J. O., and P. A. Hansen. 1996. Regulation of glucose transport into skeletal muscle, Reviews of Physiology Biochemistry and Pharmacology, Volume 128: Volume: 128. Springer Berlin Heidelberg, Berlin, Heidelberg. p. 99-193.

Holzer, P. 1988. Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. Neuroscience 24(3):739-768. doi: 10.1016/0306-4522(88)90064-4

Horst, R. L., H. F. Deluca, and N. A. Jorgensen. 1978. The effect of age on calcium absorption and accumulation of 1,25-hydroxyvitamin D3 in intestinal mucosa of rats. Metabolic Bone Disease and Related Research 1(1):29-33. doi: 10.1016/0221-8747(78)90033-4

Hoyda, T., W. Samson, and A. Ferguson. 2012. Central system roles for adiponectin in neuroendocrine and automic function. Adipokines, Science Publishers, CRC Press, Boca Raton, FL, USA:167-184.

Hubbard, R. W. 1989. Heatstroke pathophysiology: the energy depletion model, ARMY RESEARCH INST OF ENVIRONMENTAL MEDICINE NATICK MA.

Hung, A. T., B. J. Leury, M. A. Sabin, C. L. Collins, and F. R. Dunshea. 2014. Dietary nano-chromium tripicolinate increases feed intake and decreases plasma cortisol in finisher gilts during summer. Tropical animal health and production 46(8):1483-1489. doi: 10.1007/s11250-014-0673-7

Imik, H., H. Ozlu, R. Gumus, M. A. Atasever, S. Urcar, and M. Atasever. 2012. Effects of ascorbic acid and α-lipoic acid on performance and meat quality of broilers subjected to heat stress. British Poultry Science 53(6):800-808. doi: 10.1080/00071668.2012.740615

Ingram, D. 1965. Evaporative cooling in the pig. Nature 207(4995):415-416.

Itoh, F., Y. Obara, M. T. Rose, H. Fuse, and H. Hashimoto. 1998. Insulin and glucagon secretion in lactating cows during heat exposure. Journal of animal science 76(8):2182-2189.

Iwai, K., A. Yazawa, and T. Watanabe. 2003. Roles as metabolic regulators of the non-nutrients, capsaicin and capsiate, supplemented to diets. Proceedings of the Japan Academy, Series B 79(7):207-212. doi: 10.2183/pjab.79B.207

Iwasaki, S., T. Kawamata, and Y. Tomari. 2009. Drosophila argonaute1 and argonaute2 employ distinct mechanisms for translational repression. Molecular cell 34(1):58-67.

Jacob, S. T. 1995. Regulation of ribosomal gene transcription. Biochemical Journal 306(Pt 3):617. doi: 10.1042/bj3060617

Jeanplong, F., J. Bass, H. Smith, S. Kirk, R. Kambadur, M. Sharma, and J. Oldham. 2003. Prolonged underfeeding of sheep increases myostatin and myogenic regulatory factor Myf-5 in skeletal muscle while IGF-I and myogenin are repressed. Journal of Endocrinology 176(3):425. doi: 10.1677/joe.0.1760425

Jendza, J. A., R. N. Dilger, S. A. Adedokun, J. S. Sands, and O. Adeola. 2005. Escherichia coli phytase improves growth performance of starter, grower, and finisher pigs fed phosphorus-deficient diets. J Anim Sci 83(8):1882-1889. doi: 10.2527/2005.8381882x

Jensen, M. S., S. K. Jensen, and K. Jakobsen. 1997. Development of digestive enzymes in pigs with emphasis on lipolytic activity in the stomach and pancreas. Journal of Animal Science 75(2):437-445. doi: 10.2527/1997.752437x

Jentjens, R. L., A. J. Wagenmakers, and A. E. Jeukendrup. 2002. Heat stress increases muscle glycogen use but reduces the oxidation of ingested carbohydrates during exercise. Journal of applied physiology 92(4):1562-1572. doi: 10.1152/japplphysiol.00482.2001.

Jones, N. C., K. J. Tyner, L. Nibarger, H. M. Stanley, D. D. W. Cornelison, Y. V. Fedorov, and B. B. Olwin. 2005. The p38alpha/beta MAPK functions as a molecular switch to activate the quiescent satellite cell. The Journal of cell biology 169(1):105-116. doi: 10.1083/jcb.200408066

Kadim, I., O. Mahgoub, W. Al-Marzooqi, D. Al-Ajmi, R. Al-Maqbali, and S. Al-Lawati. 2008. The influence of seasonal temperatures on meat quality characteristics of hot-boned, m. psoas major and minor, from goats and sheep. Meat science 80(2):210-215. doi: 10.1016/j.meatsci.2007.11.022.

Karbach, U. 1992. Paracellular calcium transport across the small intestine. The Journal of nutrition 122(suppl_3):672-677. doi: 10.1093/jn/122.suppl_3.672

Katsumata, M., H. Yano, N. Ishida, and A. Miyazaki. 1990. Influence of a high ambient temperature and administration of clenbuterol on body composition in rats. Journal of nutritional science and vitaminology 36(6):569-578. doi: 10.3177/jnsv.36.569

Kawada, T., S.-I. Sakabe, T. Watanabe, M. Yamamoto, and K. Iwai. 1988. Some pungent principles of spices cause the adrenal medulla to secrete catecholamine in anesthetized rats. Proceedings of the Society for Experimental Biology and Medicine 188(2):229-233. doi: 10.3181/00379727-188-2-RC1

Kawada, T., T. Suzuki, M. Takahashi, and K. Iwai. 1984. Gastrointestinal absorption and metabolism of capsaicin and dihydrocapsaicin in rats. Toxicology and applied pharmacology 72(3):449-456. doi: 10.1016/0041-008X(84)90121-2

Kebreab, E., and D. Vitti. 2005. Mineral metabolism. Quantitative Aspects of Ruminant Digestion and Metabolism. CAB International, Wallingford, UK:469-486.

Kerr, B. J., and R. A. Easter. 1995. Effect of feeding reduced protein, amino acid-supplemented diets on nitrogen and energy balance in grower pigs. Journal of Animal Science 73(10):3000-3008. doi: 10.2527/1995.73103000x

Kerr, B. J., F. K. McKeith, and R. A. Easter. 1995. Effect on performance and carcass characteristics of nursery to finisher pigs fed reduced crude protein, amino acid-supplemented diets. Journal of Animal Science 73(2):433-440. doi: 10.2527/1995.732433x

Khvorova, A., A. Reynolds, and S. D. Jayasena. 2003. Functional siRNAs and miRNAs exhibit strand bias. Cell 115(2):209-216.

Kim, D. K., H. S. Lillehoj, S. H. Lee, S. I. Jang, and D. Bravo. 2010. High-throughput gene expression analysis of intestinal intraepithelial lymphocytes after oral feeding of carvacrol, cinnamaldehyde, or Capsicum oleoresin. Poultry science 89(1):68-81.

Kim, H. K., Y. S. Lee, U. Sivaprasad, A. Malhotra, and A. Dutta. 2006. Muscle-specific microRNA miR-206 promotes muscle differentiation. The Journal of cell biology 174(5):677-687.

Kim, Y. H. B., R. D. Warner, and K. Rosenvold. 2014. Influence of high pre-rigor temperature and fast pH fall on muscle proteins and meat quality: a review. Animal Production Science 54(4):375-395.

Koplas, P. A., R. L. Rosenberg, and G. S. Oxford. 1997. The Role of Calcium in the Desensitization of Capsaicin Responses in Rat Dorsal Root Ganglion Neurons. The Journal of Neuroscience 17(10):3525-3537. doi: 10.1523/jneurosci.17-10-03525.1997

Kouba, M., D. Hermier, and J. Le Dividich. 2001. Influence of a high ambient temperature on lipid metabolism in the growing pig. Journal of Animal Science 79(1):81-87. doi: 10.2527/2001.79181x.

Krol, J., K. Sobczak, U. Wilczynska, M. Drath, A. Jasinska, D. Kaczynska, and W. J. Krzyzosiak. 2004. Structural features of microRNA (miRNA) precursors and their relevance to miRNA biogenesis and small interfering RNA/short hairpin RNA design. Journal of Biological Chemistry 279(40):42230-42239.

Kuang, S., K. Kuroda, F. Le Grand, and M. A. Rudnicki. 2007. Asymmetric Self-Renewal and Commitment of Satellite Stem Cells in Muscle. Cell 129(5):999-1010. doi: 10.1016/j.cell.2007.03.044

Kunz, W. S. 2001. Control of oxidative phosphorylation in skeletal muscle. Biochim Biophys Acta 1504(1):12-19. doi: 10.1016/s0005-2728(00)00235-8

Lagos-Quintana, M., R. Rauhut, A. Yalcin, J. Meyer, W. Lendeckel, and T. Tuschl. 2002. Identification of tissue-specific microRNAs from mouse. Current biology 12(9):735-739.

Lambert, G. P. 2009. Stress-induced gastrointestinal barrier dysfunction and its inflammatory effects. Journal of animal science 87(suppl_14):E101-E108.

Le Bellego, L., and J. Noblet. 2002. Performance and utilization of dietary energy and amino acids in piglets fed low protein diets. Livestock Production Science 76(1):45-58. doi: 10.1016/S0301-6226(02)00008-8

Le Dividich, J., M. Vermorel, J. Noblet, J. Bouvier, and A. Aumaitre. 1980. Effects of environmental temperature on heat production, energy retention, protein and fat gain in early weaned piglets. British Journal of Nutrition 44(3):313-323. doi: 10.1079/BJN19800046

Lee, Y., C. Ahn, J. Han, H. Choi, J. Kim, J. Yim, J. Lee, P. Provost, O. Rådmark, and S. Kim. 2003. The nuclear RNase III Drosha initiates microRNA processing. Nature 425(6956):415.

Lei, Y., J. K. Kim, H. N. Tran, and I. H. Kim. 2017. Effect of feed flavor and sweetener on growth performance, nutrient digestibility, blood profile, and diarrhea score in weaning pigs. Korean Journal of Agricultural Science 44(1):77-85.

Lepper, C., T. A. Partridge, and C.-M. Fan. 2011. An absolute requirement for Pax7-positive satellite cells in acute injury-induced skeletal muscle regeneration. Development 138(17):3639-3646. doi: 10.1242/dev.067595

Lewis, B. P., C. B. Burge, and D. P. Bartel. 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. cell 120(1):15-20.

Lewis, B. P., I.-h. Shih, M. W. Jones-Rhoades, D. P. Bartel, and C. B. Burge. 2003. Prediction of mammalian microRNA targets. Cell 115(7):787-798.

Liu, F., P. Celi, S. Chauhan, J. Cottrell, A. Abrasaldo, S. Talukder, B. Leury, and F. Dunshea. 2015a. Effects of heat stress and antioxidants (selenium or vitamin E) supplementation on oxidative status in growing pigs. In: Proceedings of American Dairy Science Association and American Society of Animal Science midwest meeting'.(American Dairy Science Association and American Society of Animal Science: Des Moines, IA)

Liu, F., J. Cottrell, D. Wijesiriwardana, F. Kelly, P. Celi, B. Leury, and F. Dunshea. 2015b. Chromium supplementation alleviates heat stress in growing pigs. In: Proceedings of ADSA-ASAS Joint Annual Meeting (JAM)'.(Eds A Kertz, D Aaron)(American Dairy Science Association and American Society of Animal Science: Orlando, FL)

Liu, F., J. J. Cottrell, J. B. Furness, L. R. Rivera, F. W. Kelly, U. Wijesiriwardana, R. V. Pustovit, L. J. Fothergill, D. M. Bravo, and P. Celi. 2016. Selenium and vitamin E together improve intestinal epithelial barrier function and alleviate oxidative stress in heat-stressed pigs. Experimental physiology 101(7):801-810. doi: 10.1113/EP085746

Liu, H., S.-E. Chen, B. Jin, J. A. Carson, A. Niu, W. Durham, J.-Y. Lai, and Y.-P. Li. 2010. TIMP3: a physiological regulator of adult myogenesis. Journal of cell science 123(17):2914-2921. doi: 10.1242/jcs.057620

Liu, J., D. W. Bollinger, D. R. Ledoux, and T. L. Venum. 2000. Effects of dietary calcium:phosphorus ratios on apparent absorption of calcium and phosphorus in the small intestine, cecum, and colon of pigs. Journal of Animal Science 78(1):106-109. doi: 10.2527/2000.781106x

Liu, L., and S. A. Simon. 1996. Capsaicin-induced currents with distinct desensitization and Ca2+ dependence in rat trigeminal ganglion cells. Journal of Neurophysiology 75(4):1503-1514. doi: 10.1152/jn.1996.75.4.1503

Liu, Y., T. Che, M. Song, J. Lee, J. Almeida, D. Bravo, W. Van Alstine, and J. Pettigrew. 2013a. Dietary plant extracts improve immune responses and growth efficiency of pigs

experimentally infected with porcine reproductive and respiratory syndrome virus. Journal of animal science 91(12):5668-5679.
Liu, Y., M. Song, T. Che, J. Almeida, J. Lee, D. Bravo, C. W. Maddox, and J. Pettigrew. 2013b. Dietary plant extracts alleviate diarrhea and alter immune responses of weaned pigs experimentally infected with a pathogenic Escherichia coli. Journal of animal science 91(11):5294-5306.
Lu, J., T. A. McKinsey, C.-L. Zhang, and E. N. Olson. 2000. Regulation of skeletal myogenesis by association of the MEF2 transcription factor with class II histone deacetylases. Molecular cell 6(2):233-244.
MacGhee, M. E., J. S. Bradley, S. R. McCoski, A. M. Reeg, A. D. Ealy, and S. E. Johnson. 2017. Plane of nutrition affects growth rate, organ size and skeletal muscle satellite cell activity in newborn calves. Journal of Animal Physiology and Animal Nutrition 101(3):475-483. doi: 10.1111/jpn.12568
Maddock, K., J. Carroll, and E. Berg. 2003. Evaluation of the potential role of alpha-lipoic acid with regard to health and performance of weanling pigs. Journal of Animal and Veterinary Advances
Maini, S., S. K. Rastogi, J. P. Korde, A. K. Madan, and S. K. Shukla. 2007. Evaluation of oxidative stress and its amelioration through certain antioxidants in broilers during summer. The Journal of Poultry Science 44(3):339-347. doi: 10.2141/jpsa.44.339
Marai, I., A. El-Darawany, A. Fadiel, and M. Abdel-Hafez. 2007. Physiological traits as affected by heat stress in sheep—a review. Small ruminant research 71(1-3):1-12.
Matarneh, S. K., E. M. England, T. L. Scheffler, and D. E. Gerrard. 2017. Chapter 5 - The Conversion of Muscle to Meat. In: F. Toldra´, editor, Lawrie´s Meat Science (Eighth Edition). Woodhead Publishing. p. 159-185.
Mauro, A. 1961. Satellite cell of skeletal muscle fibers. The Journal of biophysical and biochemical cytology 9(2):493. doi: 10.1083/jcb.9.2.493
McCarthy, J. J. 2011. The MyomiR network in skeletal muscle plasticity. Exercise and sport sciences reviews 39(3):150.
McCullagh, K. J., R. C. Poole, A. P. Halestrap, M. O'Brien, and A. Bonen. 1996. Role of the lactate transporter (MCT1) in skeletal muscles. American Journal of Physiology-Endocrinology and Metabolism 271(1):E143-E150. doi: 10.1152/ajpendo.1996.271.1.E143
McDowell, L. R. 2003. Minerals in animal and human nutrition. 2nd ed. ed. Elsevier, Amsterdam.
McGlone, J. J., W. Stansbury, and L. Tribble. 1988. Management of lactating sows during heat stress: effects of water drip, snout coolers, floor type and a high energy-density diet. Journal of Animal Science 66(4):885-891.
Mertz, W. 1993. Chromium in human nutrition: a review. The Journal of nutrition 123(4):626-633. doi: 10.1093/jn/123.4.626
Meunier, J.-P., J.-M. Cardot, E. Manzanilla, M. Wysshaar, and M. Alric. 2007. Use of spray-cooling technology for development of microencapsulated capsicum oleoresin for the growing pig as an alternative to in-feed antibiotics: a study of release using in vitro models. Journal of animal science 85(10):2699-2710. doi: 10.2527/jas.2007-0027
Minton, J. E. 1994. Function of the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system in models of acute stress in domestic farm animals. Journal of animal science 72(7):1891-1898. doi: 10.2527/1994.7271891x.

Mitlohner, F., M. Galyean, and J. McGlone. 2002. Shade effects on performance, carcass traits, physiology, and behavior of heat-stressed feedlot heifers. Journal of Animal Science 80(8):2043-2050. doi: 10.2527/2002.8082043x.

Montilla, S. I. R., T. P. Johnson, and P.-S. C. Temperature. 2014. Heat stress causes oxidative stress but not inflammatory signaling in porcine skeletal muscle. Temperature doi: 10.4161/temp.28844

Moran, A., M. Al-Rammahi, C. Zhang, D. Bravo, S. Calsamiglia, and S. Shirazi-Beechey. 2014. Sweet taste receptor expression in ruminant intestine and its activation by artificial sweeteners to regulate glucose absorption. Journal of dairy science 97(8):4955-4972. doi: 10.3168/jds.2014-8004

Moran, A. W., M. A. Al-Rammahi, D. J. Batchelor, E. A. Coulter, K. Daly, D. Bravo, and S. P. Shirazi- Beeckey. 2010. Expression of Na+/glucose co-transporter 1 (SGLT1) is enhanced by supplementation of the diet of weaning piglets with artificial sweeteners. British Journal of Nutrition doi: 10.1017/S0007114510000917

Moran, A. W., M. A. Al-Rammahi, K. Daly, E. Grand, C. Ionescu, D. M. Bravo, E. H. Wall, and S. P. Shirazi-Beechey. 2019. Consumption of a Natural High-Intensity Sweetener Enhances Activity and Expression of Rabbit Intestinal Na+/Glucose Cotransporter 1 (SGLT1) and Improves Colibacillosis-Induced Enteric Disorders. Journal of agricultural and food chemistry

Morera, P., L. Basiricò, K. Hosoda, and U. Bernabucci. 2012. Chronic heat stress up-regulates leptin and adiponectin secretion and expression and improves leptin, adiponectin and insulin sensitivity in mice. 48(2):129. doi: 10.1530/jme-11-0054

Moro, M., G. Beran, R. Griffith, and L. Hoffman. 2000. Effects of heat stress on the antimicrobial drug resistance of Escherichia coli of the intestinal flora of swine. Journal of applied microbiology 88(5):836-844. doi: 10.1046/j.1365-2672.2000.01023.xC

Moss, F., and C. Leblond. 1971. Satellite cells as the source of nuclei in muscles of growing rats. The Anatomical Record 170(4):421-435.

Mujahid, A., N. R. Pumford, W. Bottje, K. Nakagawa, T. Miyazawa, Y. Akiba, and M. Toyomizu. 2007. Mitochondrial oxidative damage in chicken skeletal muscle induced by acute heat stress. The Journal of Poultry Science 44(4):439-445. doi: 10.2141/jpsa.44.439.

Mujahid, A., K. Sato, Y. Akiba, and M. Toyomizu. 2006. Acute heat stress stimulates mitochondrial superoxide production in broiler skeletal muscle, possibly via downregulation of uncoupling protein content. Poultry science 85(7):1259-1265. doi: 10.1093/ps/85.7.1259

Nardone, A., B. Ronchi, N. Lacetera, and U. Bernabucci. 2006. Climatic effects on productive traits in livestock. Veterinary Research Communications 30:75-81.

Nardone, A., B. Ronchi, N. Lacetera, M. S. Ranieri, and U. Bernabucci. 2010. Effects of climate changes on animal production and sustainability of livestock systems. Livestock Science 130(1-3):57-69.

Näsi, M. 1990. Microbial phytase supplementation for improving availability of plant phosphorus in the diet of the growing pigs. Agricultural and Food Science 62(5):435-443. doi: 10.23986/afsci.72917

Nevius, E., P. Srivastava, and S. Basu. 2012. Oral ingestion of capsaicin, the pungent component of chili pepper, enhances a discreet population of macrophages and confers protection from autoimmune diabetes. Mucosal immunology 5(1):76. doi: 10.1038/mi.2011.50

Nielsen, S., C. Scheele, C. Yfanti, T. Åkerström, A. R. Nielsen, B. K. Pedersen, and M. Laye. 2010. Muscle specific microRNAs are regulated by endurance exercise in human skeletal muscle. The Journal of physiology 588(20):4029-4037. doi: 10.1113/jphysiol.2010.189860

Nienaber, J., G. Hahn, R. Eigenberg, R. Korthals, J. T. Yen, and D. Harris. 1997. Genetic and heat stress interaction effects on finishing swine. Livestock environment V 2

O'Neill, L. A. 2011. A critical role for citrate metabolism in LPS signalling. Biochemical Journal 438(3):e5-e6. doi: 10.1042/BJ20111386

O'Rourke, J. R., S. A. Georges, H. R. Seay, S. J. Tapscott, M. T. McManus, D. J. Goldhamer, M. S. Swanson, and B. D. Harfe. 2007. Essential role for Dicer during skeletal muscle development. Developmental biology 311(2):359-368.

O'brien, M., R. Rhoads, S. Sanders, G. Duff, and L. Baumgard. 2010. Metabolic adaptations to heat stress in growing cattle. Domestic animal endocrinology 38(2):86-94. doi: 10.1016/j.domaniend.2009.08.005

Okoruwa, M. 2014. Effect of heat stress on thermoregulatory, live bodyweight and physiological responses of dwarf goats in southern Nigeria. European Scientific Journal 10(27)

Olguin, H. C., and B. B. Olwin. 2004. Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal. Dev Biol 275(2):375-388. doi: 10.1016/j.ydbio.2004.08.015

Ontjes, D. A. 2011. 6 Hormone Actions in the Regulation of Calcium and Phosphorus Metabolism. Diet, Nutrients, and Bone Health:71-112.

Oresanya, T., A. Beaulieu, and J. Patience. 2008. Investigations of energy metabolism in weanling barrows: The interaction of dietary energy concentration and daily feed (energy) intake. Journal of animal science 86(2):348-363. doi: 10.2527/jas.2007-0009

Orrenius, S., B. Zhivotovsky, and P. Nicotera. 2003. Regulation of cell death: the calcium–apoptosis link. Nature reviews Molecular cell biology 4(7):552-565. doi: 10.1038/nrm1150

Owens, C., C. Alvarado, and A. Sams. 2009. Research developments in pale, soft, and exudative turkey meat in North America. Poultry Science 88(7):1513-1517.

Packer, L., E. H. Witt, and H. J. Tritschler. 1995. Alpha-lipoic acid as a biological antioxidant. Free radical biology and medicine 19(2):227-250. doi: 10.1016/0891-5849(95)00017-R

Palo, P. E., J. L. Sell, F. J. Piquer, M. F. Soto-Salanova, and L. Vilaseca. 1995. Effect of Early Nutrient Restriction on Broiler Chickens.: 1. Performance and Development of the Gastrointestinal Tract1. Poultry Science 74(1):88-101. doi: 10.3382/ps.0740088

Partridge, I. 1978. Studies on digestion and absorption in the intestines of growing pigs: 3. Net movements of mineral nutrients in the digestive tract. British Journal of Nutrition 39(3):527-537. doi: 10.1079/BJN19780068

Pastorelli, G., M. Neil, and I. Wigren. 2009. Body composition and muscle glycogen contents of piglets of sows fed diets differing in fatty acids profile and contents. Livestock science 123(2-3):329-334. doi: 10.1016/j.livsci.2008.11.023

Pearce, S., V. Mani, T. Weber, R. Rhoads, J. Patience, L. Baumgard, and N. Gabler. 2013a. Heat stress and reduced plane of nutrition decreases intestinal integrity and function in pigs. Journal of Animal Science 91(11):5183-5193. doi: 10.1371/journal.pone.0070215

Pearce, S., M.-V. Sanz Fernandez, J. Torrison, M. Wilson, L. Baumgard, and N. Gabler. 2015. Dietary organic zinc attenuates heat stress–induced changes in pig intestinal integrity and metabolism. Journal of Animal Science 93(10):4702-4713. doi: 10.2527/jas.2015-9018

Pearce, S. C., N. K. Gabler, J. W. Ross, J. Escobar, J. F. Patience, R. P. Rhoads, and L. H. Baumgard. 2013b. The effects of heat stress and plane of nutrition on metabolism in growing pigs. Journal of animal science 91(5):2108-2118. doi: 10.2527/jas.2012-5738

Pearce, S. C., V. Mani, R. L. Boddicker, J. S. Johnson, T. E. Weber, J. W. Ross, R. P. Rhoads, L. H. Baumgard, and N. K. Gabler. 2013c. Heat stress reduces intestinal barrier integrity and favors intestinal glucose transport in growing pigs. PloS one 8(8):e70215.

Pettey, L., G. Cromwell, and M. Lindemann. 2006. Estimation of endogenous phosphorus loss in growing and finishing pigs fed semi-purified diets. Journal of animal science 84(3):618-626. doi: 10.2527/2006.843618x

Pfeffer, E., D. Beede, and H. Valk. 2005. Phosphorus metabolism in ruminants and requirements of cattle. Nitrogen and Phosphorus Nutrition of Cattle. CAB International, Wallingford, UK:195-231.

Platel, K., and K. Srinivasan. 2000. Influence of dietary spices and their active principles on pancreatic digestive enzymes in albino rats. Food/Nahrung 44(1):42-46. doi: 10.1002/(SICI)1521-3803(20000101)44:1<42::AID-FOOD42>3.0.CO;2-D

Pollman, D. 2010. Seasonal effects on sow herds: industry experience and management strategies. J. Anim. Sci 88(Suppl 3):9.

Pond, W., D. Church, K. Pond, and P. Schoknecht. 2005. Macromineral elements. Pond WG, Church DC, Pond KR, Schoknecht PA. Basic Animal Nutrition and Feeding:164-183.

Pragna, P., V. Sejian, N. Soren, M. Bagath, G. Krishnan, V. Beena, P. I. Devi, and R. Bhatta. 2018. Summer season induced rhythmic alterations in metabolic activities to adapt to heat stress in three indigenous (Osmanabadi, Malabari and Salem Black) goat breeds. Biological Rhythm Research 49(4):551-565.

Qu, H., and K. Ajuwon. 2018. Adipose tissue-specific responses reveal an important role of lipogenesis during heat stress adaptation in pigs. Journal of animal science 96(3):975-989. doi: 10.1093/jas/sky022.

Rachagani, S., Y. Cheng, and J. M. Reecy. 2010. Myostatin genotype regulates muscle-specific miRNA expression in mouse pectoralis muscle. BMC Research Notes 3(1):297. doi: 10.1186/1756-0500-3-297

Rao, P. K., R. M. Kumar, M. Farkhondeh, S. Baskerville, and H. F. Lodish. 2006. Myogenic factors that regulate expression of muscle-specific microRNAs. Proceedings of the National Academy of Sciences 103(23):8721-8726. doi: 10.1073/pnas.0602831103

Reed, L. 1974. Accounts of Chem. Res 7:40-46.

Regen, D. M., D. A. Young, W. W. Davis, J. Jack, Jr., and C. R. Park. 1964. Adjustment of glycolysis to energy utilization in the perfused rat heart. The effect of changes in the ionic composition of the medium on phosphofructokinase activity. J Biol Chem 239:381-384.

Reinbach, H. C. 2008. Hot spices: effects on appetite, energy intake and sensory properties of a meal, University of Copenhagen, Faculty of Life Sciences [Department of Food ….

Reinhart, B. J., F. J. Slack, M. Basson, A. E. Pasquinelli, J. C. Bettinger, A. E. Rougvie, H. R. Horvitz, and G. Ruvkun. 2000. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. nature 403(6772):901.

Relaix, F., D. Montarras, S. Zaffran, B. Gayraud-Morel, D. Rocancourt, S. Tajbakhsh, A. Mansouri, A. Cumano, and M. Buckingham. 2006. Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells. J Cell Biol 172(1):91-102.

Renaudeau, D., A. Collin, S. Yahav, V. De Basilio, J. Gourdine, and R. J. Collier. 2012. Adaptation to hot climate and strategies to alleviate heat stress in livestock production. Animal 6(5):707-728. doi: 10.1017/S1751731111002448

Renaudeau, D., J. Gourdine, B. Silva, and J. Noblet. 2008. Nutritional routes to attenuate heat stress in pigs. Livestock and Global Climate Change:134.

Renter, D. G., D. R. Smith, R. King, R. Stilborn, J. Berg, J. Berezowski, and M. McFall. 2008. Detection and determinants of Escherichia coli O157: H7 in Alberta feedlot pens immediately prior to slaughter. Canadian Journal of Veterinary Research 72(3):217.

Rhoads, M., R. Rhoads, M. VanBaale, R. J. Collier, S. Sanders, W. J. Weber, B. A. Crooker, and L. Baumgard. 2009. Effects of heat stress and plane of nutrition on lactating Holstein cows: I. Production, metabolism, and aspects of circulating somatotropin. Journal of dairy science 92(5):1986-1997.

Rhoads, R. P., L. H. Baumgard, J. K. Suagee, and S. R. Sanders. 2013. Nutritional interventions to alleviate the negative consequences of heat stress. Advances in nutrition 4(3):267-276. doi: 10.3945/an.112.003376

Ribeiro, C. B., D. C. Christofoletti, V. A. Pezolato, R. de Cássia Marqueti Durigan, J. Prestes, R. A. Tibana, E. C. L. Pereira, I. V. de Sousa Neto, J. L. Q. Durigan, and C. A. da Silva. 2015. Leucine minimizes denervation-induced skeletal muscle atrophy of rats through akt/mtor signaling pathways. Frontiers in Physiology 6(73)(Original Research) doi: 10.3389/fphys.2015.00073

Roach, P. J. 1990. Control of glycogen synthase by hierarchal protein phosphorylation. The FASEB Journal 4(12):2961-2968. doi: 10.1096/fasebj.4.12. 2168324.

Röder, P. V., K. E. Geillinger, T. S. Zietek, B. Thorens, H. Koepsell, and H. Daniel. 2014. The Role of SGLT1 and GLUT2 in Intestinal Glucose Transport and Sensing. PLOS ONE 9(2):e89977. doi: 10.1371/journal.pone.0089977

Romijn, J. A., E. F. Coyle, L. S. Sidossis, A. Gastaldelli, J. F. Horowitz, E. Endert, and R. R. Wolfe. 1993. Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration. American Journal of Physiology-Endocrinology and Metabolism 265(3):E380-E391. doi: 10.1152/ajpendo.1993.265.3.E380

Ronchi, B., N. Lacetera, U. Bernabucci, A. Nardone, and A. Verini Supplizi. 1999. Distinct and common effects of heat stress and restricted feeding on metabolic status of Holstein heifers. Zootecnica e Nutrizione Animale (Italy)

Rosenberg, M. I., S. A. Georges, A. Asawachaicharn, E. Analau, and S. J. Tapscott. 2006. MyoD inhibits Fstl1 and Utrn expression by inducing transcription of miR-206. The Journal of cell biology 175(1):77-85. doi: 10.1083/jcb.200603039

Ryall, J. G. 2013. Metabolic reprogramming as a novel regulator of skeletal muscle development and regeneration. Febs j 280(17):4004-4013. doi: 10.1111/febs.12189

Ryder, A., J. Feddes, and M. Zuidhof. 2004. Field study to relate heat stress index to broiler performance. Journal of applied poultry research 13(3):493-499. doi: 10.1093/japr/13.3.493

Samanta, S., S. Haldar, V. Bahadur, and T. K. Ghosh. 2008. Chromium picolinate can ameliorate the negative effects of heat stress and enhance performance, carcass and meat traits in broiler chickens by reducing the circulatory cortisol level. Journal of the Science of Food and Agriculture 88(5):787-796. doi: 10.1002/jsfa.3146

Sanders, S. R., L. C. Cole, K. L. Flann, L. H. Baumgard, and R. P. Rhoads. 2009. Effects of acute heat stress on skeletal muscle gene expression associated with energy metabolism in rats. Federation of American Societies for Experimental Biology.

Sandow, A. 1952. Excitation-contraction coupling in muscular response. Yale J Biol Med 25(3):176-201.

Sano, H., K. Takahashi, K. Ambo, and T. Tsuda. 1983. Turnover and oxidation rates of blood glucose and heat production in sheep exposed to heat. Journal of Dairy Science 66(4):856-861. doi: 10.3168/jds.S0022-0302(83)81867-0

Santos, C., J. Almeida, E. Matias, M. Fraqueza, C. Roseiro, and L. Sardina. 1997. Influence of lairage environmental conditions and resting time on meat quality in pigs. Meat science 45(2):253-262.

Sanz Fernandez, M. V., S. K. Stoakes, M. Abuajamieh, J. T. Seibert, J. S. Johnson, E. A. Horst, R. P. Rhoads, and L. H. Baumgard. 2015. Heat stress increases insulin sensitivity in pigs. Physiological reports 3(8):e12478. doi: 10.14814/phy2.12478

Schiaffino, S., and C. Mammucari. 2011. Regulation of skeletal muscle growth by the IGF1-Akt/PKB pathway: insights from genetic models. Skeletal Muscle 1(1):4. doi: 10.1186/2044-5040-1-4

Schwarz, D. S., G. Hutvágner, B. Haley, and P. D. Zamore. 2002. Evidence that siRNAs function as guides, not primers, in the Drosophila and human RNAi pathways. Molecular cell 10(3):537-548.

Seale, P., S. Kajimura, W. Yang, S. Chin, L. M. Rohas, M. Uldry, G. Tavernier, D. Langin, and B. M. Spiegelman. 2007. Transcriptional control of brown fat determination by PRDM16. Cell metabolism 6(1):38-54. doi: 10.1016/j.cmet.2007.06.001

Seale, P., L. A. Sabourin, A. Girgis-Gabardo, A. Mansouri, P. Gruss, and M. A. Rudnicki. 2000. Pax7 is required for the specification of myogenic satellite cells. Cell 102(6):777-786. doi: 10.1016/s0092-8674(00)00066-0

Self, J., R. Luna-Gierke, A. Fothergill, K. Holt, and A. Vieira. 2017. Outbreaks attributed to pork in the United States, 1998–2015. Epidemiology & Infection 145(14):2980-2990. doi: 10.1017/S0950268817002114

Selle, P. H., and V. Ravindran. 2008. Phytate-degrading enzymes in pig nutrition. Livestock Science 113(2-3):99-122. doi: 10.1016/j.livsci.2007.05.014

Shapiro, R., and R. P. Heaney. 2003. Co-dependence of calcium and phosphorus for growth and bone development under conditions of varying deficiency. Bone 32(5):532-540. doi: 10.1016/S8756-3282(03)00060-7

Sharkey, K., R. Williams, and G. Dockray. 1984. Sensory substance P innervation of the stomach and pancreas: demonstration of capsaicin-sensitive sensory neurons in the rat by combined immunohistochemistry and retrograde tracing. Gastroenterology 87(4):914-921. doi: 10.1016/0016-5085(84)90088-X

Shi, X., F. Zhou, X. Li, B. Chang, D. Li, Y. Wang, Q. Tong, Y. Xu, M. Fukuda, and J. J. Zhao. 2013. Central GLP-2 enhances hepatic insulin sensitivity via activating PI3K signaling in POMC neurons. Cell metabolism 18(1):86-98.

Shwartz, G., M. Rhoads, M. VanBaale, R. Rhoads, and L. Baumgard. 2009. Effects of a supplemental yeast culture on heat-stressed lactating Holstein cows. Journal of dairy science 92(3):935-942. doi: 10.3168/jds.2008-1496

Siems, W., W. Dubiel, R. Dumdey, M. Müller, and S. M. Rapoport. 1984. Accounting for the ATP-consuming processes in rabbit reticulocytes. European Journal of Biochemistry 139(1):101-107. doi: 10.1111/j.1432-1033.1984.tb07982.x

Silva, B., R. Oliveira, J. Donzele, H. Fernandes, M. Abreu, J. Noblet, and C. Nunes. 2006. Effect of floor cooling on performance of lactating sows during summer. Livestock Science 105(1-3):176-184.

Skarp, C., M.-L. Hänninen, and H. Rautelin. 2016. Campylobacteriosis: the role of poultry meat. Clinical Microbiology and Infection 22(2):103-109. doi: 10.1016/j.cmi.2015.11.019.

Soares Jr, J. H. 1995a. Calcium bioavailability, Bioavailability of nutrients for animals. Elsevier. p. 95-118.

Soares Jr, J. H. 1995b. Phosphorus bioavailability, Bioavailability of Nutrients for Animals. Elsevier. p. 257-294.

Soltan, M. A. 2010. Effect of dietary chromium supplementation on productive and reproductive performance of early lactating dairy cows under heat stress. Journal of Animal Physiology and Animal Nutrition 94(2):264-272. doi: 10.1111/j.1439-0396.2008.00913.x

St-Pierre, N., B. Cobanov, and G. Schnitkey. 2003. Economic losses from heat stress by US livestock industries. Journal of dairy science 86:E52-E77. doi: 10.3168/jds.S0022-0302(03)74040-5

Stephens, J., B. Stoll, J. Cottrell, X. Chang, M. Helmrath, and D. G. Burrin. 2006. Glucagon-like peptide-2 acutely increases proximal small intestinal blood flow in TPN-fed neonatal piglets. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 290(2)doi: 10.1152/ajpregu.00588.2005

Sternini, C., J. Reeve, and N. Brecha. 1987. Distribution and characterization of calcitonin gene-related peptide immunoreactivity in the digestive system of normal and capsaicin-treated rats. Gastroenterology 93(4):852-862.

Streffer, C. 1988. Aspects of metabolic change after hyperthermia, Application of Hyperthermia in the Treatment of Cancer. Springer. p. 7-16.

Summers, M., B. McBride, and L. Milligan. 1988. Components of basal energy expenditure. doi: 10.7591/9781501745713-015

Sun, Q., Y. Zhang, G. Yang, X. Chen, Y. Zhang, G. Cao, J. Wang, Y. Sun, P. Zhang, and M. Fan. 2008. Transforming growth factor-β-regulated miR-24 promotes skeletal muscle differentiation. Nucleic acids research 36(8):2690-2699. doi: 10.1093/nar/gkn032

Sweetman, D., K. Goljanek, T. Rathjen, S. Oustanina, T. Braun, T. Dalmay, and A. Münsterberg. 2008. Specific requirements of MRFs for the expression of muscle specific microRNAs, miR-1, miR-206 and miR-133. Developmental biology 321(2):491-499. doi: 10.1016/j.ydbio.2008.06.019

Tabiri, H. Y., K. Sato, K. Takahashi, M. Toyomizu, and Y. Akiba. 2000. Effect of acute heat stress on plasma amino acids concentration of broiler chickens. Japanese poultry science 37(2):86-94. doi: 10.2141/jpsa.37.86

Tatsumi, R., J. E. Anderson, C. J. Nevoret, O. Halevy, and R. E. Allen. 1998. HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells. Developmental biology 194(1):114-128. doi: 10.1006/dbio.1997.8803

Tatsumi, R., S. Sheehan, H. Iwasaki, A. Hattori, and R. E. Allen. 2001. Mechanical stretch induces activation of skeletal muscle satellite cells in vitro. Experimental cell research 267(1):107-114. doi: 10.1006/excr.2001.5252

Temim, S., A.-M. Chagneau, R. Peresson, and S. Tesseraud. 2000. Chronic heat exposure alters protein turnover of three different skeletal muscles in finishing broiler chickens fed 20 or 25% protein diets. The Journal of nutrition 130(4):813-819. doi: 10.1093/jn/130.4.813

Toghyani, M., M. Toghyani, M. Shivazad, A. Gheisari, and R. Bahadoran. 2012. Chromium Supplementation Can Alleviate the Negative Effects of Heat Stress on Growth Performance, Carcass Traits, and Meat Lipid Oxidation of Broiler Chicks without Any Adverse Impacts on Blood Constituents. Biological Trace Element Research 146(2):171-180. doi: 10.1007/s12011-011-9234-3

Torlińska, T., R. Banach, J. Paluszak, and A. Gryczka-Dziadecka. 1987. Hyperthermia effect on lipolytic processes in rat blood and adipose tissue. Acta Physiologica Polonica 38(4):361-366.

Trajkovski, M., K. Ahmed, C. C. Esau, and M. Stoffel. 2012. MyomiR-133 regulates brown fat differentiation through Prdm16. Nature cell biology 14(12):1330-1335. doi: 10.1038/ncb2612

Turrens, J. F. 2003. Mitochondrial formation of reactive oxygen species. The Journal of physiology 552(2):335-344. doi: 10.1111/j.1469-7793.2003.00335.x

Van Milgen, J., and J. Noblet. 2003. Partitioning of energy intake to heat, protein, and fat in growing pigs. Journal of Animal Science 81(14_suppl_2):E86-E93. doi: 10.2527/2003.8114_suppl_2E86x

Van Rooij, E., and E. N. Olson. 2007. MicroRNAs: powerful new regulators of heart disease and provocative therapeutic targets. The Journal of clinical investigation 117(9):2369-2376.

Van Rooij, E., D. Quiat, B. A. Johnson, L. B. Sutherland, X. Qi, J. A. Richardson, R. J. Kelm Jr, and E. N. Olson. 2009. A family of microRNAs encoded by myosin genes governs myosin expression and muscle performance. Developmental cell 17(5):662-673. doi: 10.1016/j.devcel.2009.10.013

Varasteh, S., J. Fink-Gremmels, J. Garssen, and S. Braber. 2018. α-Lipoic acid prevents the intestinal epithelial monolayer damage under heat stress conditions: model experiments in Caco-2 cells. European Journal of Nutrition 57(4):1577-1589. doi: 10.1007/s00394-017-1442-y

Vegge, A., T. Thymann, P. Lund, B. Stoll, S. B. Bering, B. Hartmann, J. Jelsing, N. Qvist, D. G. Burrin, and P. B. Jeppesen. 2013. Glucagon-like peptide-2 induces rapid digestive adaptation following intestinal resection in preterm neonates. American Journal of Physiology-Gastrointestinal and Liver Physiology 305(4):G277-G285. doi: 10.1152/ajpgi.00064.2013

Verstegen, M., E. Brascamp, and W. V. D. HEL. 1978. Growing and fattening of pigs in relation to temperature of housing and feeding level. Canadian Journal of Animal Science 58(1):1-13. doi: 10.4141/cjas78-001

Veum, T., and M. Ellersieck. 2008. Effect of low doses of Aspergillus niger phytase on growth performance, bone strength, and nutrient absorption and excretion by growing and finishing swine fed corn-soybean meal diets deficient in available phosphorus and calcium. Journal of animal science 86(4):858-870. doi: 10.2527/jas.2007-0312

Veum, T., D. Ledoux, and V. Raboy. 2007. Low-phytate barley cultivars improve the utilization of phosphorus, calcium, nitrogen, energy, and dry matter in diets fed to young swine. Journal of animal science 85(4):961-971. doi: 10.2527/jas.2006-453

Vier, C. M., F. Wu, S. S. Dritz, M. D. Tokach, M. A. D. Goncalves, U. A. D. Orlando, J. C. Woodworth, R. D. Goodband, and J. M. DeRouchey. 2017. 119 Standardized total tract

digestible phosphorus requirement of 11- to 25-kg pigs. Journal of Animal Science 95(suppl_2):56-56. doi: 10.2527/asasmw.2017.119
Wang, R., X. Pan, and Z. Peng. 2009. Effects of heat exposure on muscle oxidation and protein functionalities of pectoralis majors in broilers. Poultry Science 88(5):1078-1084. doi: 10.3382/ps.2008-00094.
Wang, X. H. 2013. MicroRNA in myogenesis and muscle atrophy. Current opinion in clinical nutrition and metabolic care 16(3):258. doi: 10.1097/MCO.0b013e32835f81b9
Wang, Y. X., and M. A. Rudnicki. 2012. Satellite cells, the engines of muscle repair. Nature Reviews Molecular Cell Biology 13(2):127-133. doi: 10.1038/nrm3265
Warner, R., F. Dunshea, D. Gutzke, J. Lau, and G. Kearney. 2014. Factors influencing the incidence of high rigor temperature in beef carcasses in Australia. Animal Production Science 54(4):363-374.
Watanabe, T., T. Kawada, T. Kato, T. Harada, and K. Iwai. 1994. Effects of capsaicin analogs on adrenal catecholamine secretion in rats. Life Sciences 54(5):369-374. doi: 10.1016/0024-3205(94)00793-4
Webster, Micah T., U. Manor, J. Lippincott-Schwartz, and C.-M. Fan. 2016. Intravital Imaging Reveals Ghost Fibers as Architectural Units Guiding Myogenic Progenitors during Regeneration. Cell Stem Cell 18(2):243-252. doi: 10.1016/j.stem.2015.11.005
West, J. W. 2003. Effects of heat-stress on production in dairy cattle. Journal of dairy science 86(6):2131-2144. doi: 10.3168/jds.S0022-0302(03)73803-X
Wheelock, J., R. Rhoads, M. VanBaale, S. Sanders, and L. Baumgard. 2010. Effects of heat stress on energetic metabolism in lactating Holstein cows. Journal of dairy science 93(2):644-655.
Wilkins, I. A., and D. W. Wheeler. 2004. Regulation of temperature. Surgery (Oxford) 22(7):168. doi: 10.1383/surg.22.7.168a.38354
Winbanks, C. E., B. Wang, C. Beyer, P. Koh, L. White, P. Kantharidis, and P. Gregorevic. 2011. TGF-β regulates miR-206 and miR-29 to control myogenic differentiation through regulation of HDAC4. Journal of Biological Chemistry 286(16):13805-13814. doi: 10.1074/jbc.M110.192625
Wray-Cahen, D., A. W. Bell, R. D. Boyd, D. A. Ross, D. E. Bauman, B. J. Krick, and R. J. Harrell. 1995. Nutrient Uptake by the Hindlimb of Growing Pigs Treated with Porcine Somatotropin and Insulin. The Journal of Nutrition 125(1):125-135. doi: 10.1093/jn/125.1.125
Wu, F., M. D. Tokach, S. S. Dritz, J. C. Woodworth, J. M. DeRouchey, R. D. Goodband, M. A. Gonçalves, and J. R. Bergstrom. 2018a. Effects of dietary calcium to phosphorus ratio and addition of phytase on growth performance of nursery pigs. Journal of animal science 96(5):1825-1837. doi: 10.1093/jas/sky101
Wu, F., J. Woodworth, M. Tokach, J. DeRouchey, S. Dritz, and R. Goodband. 2018b. Standardized total tract digestible phosphorus requirement of 13-to 28-lb pigs fed diets with or without phytase. Kansas Agricultural Experiment Station Research Reports 4(9):17.
Wu, G., D. A. Knabe, and S. W. Kim. 2004. Arginine nutrition in neonatal pigs. J Nutr 134(10 Suppl):2783S-2790S; discussion 2796S-2797S. doi: 10.1093/jn/134.10.2783S
Yamada, M., Y. Sankoda, R. Tatsumi, W. Mizunoya, Y. Ikeuchi, K. Sunagawa, and R. E. Allen. 2008. Matrix metalloproteinase-2 mediates stretch-induced activation of skeletal muscle

satellite cells in a nitric oxide-dependent manner. The international journal of biochemistry & cell biology 40(10):2183-2191. doi: 10.1016/j.biocel.2008.02.017
Yan, B., C.-D. Zhu, J.-T. Guo, L.-H. Zhao, and J.-L. Zhao. 2013. miR-206 regulates the growth of the teleost tilapia (Oreochromis niloticus) through the modulation of IGF-1 gene expression. Journal of Experimental Biology 216(7):1265-1269. doi: 10.1242/jeb.079590
Yan, Y.-E., Y.-Q. Zhao, H. Wang, and M. Fan. 2006. Pathophysiological factors underlying heatstroke. Medical hypotheses 67(3):609-617. doi: 10.1016/j.mehy.2005.12.048
Yang, C.-Y., and M.-T. Lin. 2002. Oxidative stress in rats with heatstroke-induced cerebral ischemia. Stroke 33(3):790-794. doi: 10.1161/hs0102.100208
Yang, J.-S., and E. C. Lai. 2011. Alternative miRNA biogenesis pathways and the interpretation of core miRNA pathway mutants. Molecular cell 43(6):892-903. doi: 10.1016/j.molcel.2011.07.024
Yao, J., and F. Qin. 2009. Interaction with Phosphoinositides Confers Adaptation onto the TRPV1 Pain Receptor. PLOS Biology 7(2):e1000046. doi: 10.1371/journal.pbio.1000046
Yin, H., A. Pasut, V. D. Soleimani, C. F. Bentzinger, G. Antoun, S. Thorn, P. Seale, P. Fernando, W. van IJcken, and F. Grosveld. 2013a. MicroRNA-133 controls brown adipose determination in skeletal muscle satellite cells by targeting Prdm16. Cell metabolism 17(2):210-224. doi: 10.1016/j.cmet.2013.01.004
Yin, H., F. Price, and M. A. Rudnicki. 2013b. Satellite Cells and the Muscle Stem Cell Niche. Physiological Reviews 93(1):23-67. doi: 10.1152/physrev.00043.2011
Yonei, Y., P. Holzer, and P. H. Guth. 1990. Laparotomy-induced gastric protection against ethanol injury is mediated by capsaicin-sensitive sensory neurons. Gastroenterology 99(1):3-9.
You, Y.-Q. N., P.-R. Ling, J. Z. Qu, and B. R. Bistrian. 2008. Effects of Medium-Chain Triglycerides, Long-Chain Triglycerides, or 2-Monododecanoin on Fatty Acid Composition in the Portal Vein, Intestinal Lymph, and Systemic Circulation in Rats. Journal of Parenteral and Enteral Nutrition 32(2):169-175. doi: 10.1177/0148607108314758
Yuan, Y.-R., Y. Pei, J.-B. Ma, V. Kuryavyi, M. Zhadina, G. Meister, H.-Y. Chen, Z. Dauter, T. Tuschl, and D. J. Patel. 2005. Crystal structure of A. aeolicus argonaute, a site-specific DNA-guided endoribonuclease, provides insights into RISC-mediated mRNA cleavage. Molecular cell 19(3):405-419.
Zhao, L., R. P. McMillan, G. Xie, S. G. L. W. Giridhar, L. H. Baumgard, S. W. El-Kadi, J. Selsby, J. Ross, n. Gabler, M. W. Hulver, and R. P. Rhoads. 2018. Heat stress decreases metabolic flexibility in skeletal muscle of growing pigs. Am J Physiol Regul Integr Comp Physiol doi: 10.1152/ajpregu.00404.2017

Chapter 2: Different dietary calcium and phosphorus inclusion levels alter satellite cell activity in neonatal pigs

Abstract

Postnatal skeletal muscle growth requires satellite cell activation and fusion with existing myofibers. Delay or absence of satellite cell proliferation and differentiation leads to a reduction in postnatal muscle hypertrophy and reduced growth potential. Calcium and phosphate are necessary for normal muscle development, and deficiencies cause altered satellite cell function and reduced muscle accumulation. Dietary PO_4 deficiencies were shown to cause decreased satellite cell proliferation *in vitro* while excess PO_4 caused increased proliferation. The objective of this study was to determine proliferation and differentiation of satellite cells in response to combined Ca-PO_4 deficient, adequate, and excess diets in neonatal pigs. Twenty-seven newborn piglets (24h±6h age) were assigned to dietary treatments, based on NRC requirements, containing either an adequate Ca-PO_4 available diet (CaPA), a 25% deficient Ca-PO_4 available diet (CaPD), or a 25% excess Ca-PO_4 available diet (CaPE) over a 16-day trial. Feed intake and body weight were recorded daily, and blood samples collected at 8d and 16d. Oral *bromodeoxyuridine* (BRDU) was administered 18hrs prior to sacrifice. Following sacrifice, *longissimus dorsi muscle* (LD) was collected for immunohistochemistry and satellite cell (SC) isolation. Isolated SC were cultured for *in vitro* proliferation and differentiation assays. There were no differences in average daily gain between treatments. The expression of Pax7 tended to differ between treatments (P=0.09) with CaPA pigs having 15% greater expression than CaPD pigs. CaPD pigs tended to have a 23% greater expression of Mef2a than CaPE pigs (P=0.09). Dietary deficiency reduced proliferating satellite cells (BRDU/MyoD+) *in vivo* (P=0.02) compared to CaPA and CaPE diets. However, *in vitro* SC from CaPE diets tended to proliferate 13.7% (P= 0.08) less than SC from CaPA diets with deficient diets being intermediate and not different from the two. Similarly, myoblast fusion rates were greatest in CaPA diets with deficient diets having 9.2% lower fusion rates (P= 0.05), and

CaPE diets exhibiting 20.7% lower than those fed CaPA diets (P<0.001). The progression of satellite cells through the myogenic lineage requires precise control of gene expression, in part, facilitated by micro-RNAs (miRNA) to post-transcriptionally regulate gene expression. Differentiation is stimulated by miR-206, which in this study tended to be upregulated by 9% in differentiating satellite cells from CaPA pigs than CaPD and CaPE (P=0.07). This corresponds with the increase in fusion index in CaPA pigs. These data indicate that diets deficient in Ca-PO_4 decrease satellite cell activity which may lead to a reduction in postnatal muscle growth. Additionally, CaPE appears to reduce satellite cell proliferation and differentiation *in vitro*. Diets CaPA in Ca-PO_4 have the greatest proliferation and differentiation rates which may improve the growth potential of the animal.

Introduction

While both calcium and phosphorus are critical for bone growth and development, phosphorus is also essential for the growth of all soft tissues such as muscle, fat, and connective tissue. Approximately 20% of the phosphorus found in the body is found in soft tissues in the form of phosphoproteins, phospholipids, and nucleic acids (Bertram, 1995). Even minor dietary phosphorus restriction to growing pigs reduces bone and muscle growth as well as increases body fat content (Shelton et al., 2004). Additionally, dietary phosphorus restriction has also been shown to stimulate fat accumulation in rainbow trout and crucian carp (Sugiura et al., 2011).

Early-life nutritional deficiencies of calcium and phosphorus may have lifelong consequences. Early-life nutritional restriction of muscle growth has been shown to reduce lifetime feed efficiency and alter body composition in animals (Ji et al., 2017). A potential mechanism by which early-life nutrition may impact lifelong growth outcomes is through the programming of the tissue-specific stem cells required for growth. Neonatal phosphorus deficiency altered the activity and differentiation potential of satellite cells (SCs), which are required for muscle hypertrophy during growth (Alexander et al., 2010). To date, the examination of the effect of dual deficiencies of calcium and phosphorus on the activity and functionality of tissue-specific stem cells in neonates has been conducted.

Calcium and phosphorus homeostasis is inextricably linked with and orchestrated through intestinal uptake, bone turnover, and kidney excretion and resorption. Imbalances in dietary calcium and phosphorus concentrations can have significant impacts on intestinal absorption as well as in the endocrine regulation of mineral homeostasis. In adult animals, calcium and phosphorus homeostasis is regulated by a few key hormones: parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23), calcitriol, frizzled-related protein 4, and matrix extracellular

phosphoglycoprotein. However, this endocrine regulation is not well characterized in neonates and has significant differences from what occurs in older animals (Zimmerman et al., 1960). Promoting appropriate bone and soft tissue growth in neonates is important for both human health and animal agriculture. In humans, this is done with the goal of reducing the incidence of osteoporosis and obesity, and in animal agriculture to maintain animal welfare as well as the sustainability of the production of animal products. In this study, we hypothesized that different calcium and phosphorus amounts would affect bone development and muscle growth through tissue-specific stem cell activities. We examined the impact of different dietary calcium and phosphorus concentrations in neonatal pig diets on the growth and proliferation and differentiation of MSCs and SCs.

Methods

Animals

All animal protocols were approved by University of Maryland Animal Care and Use Committee. A total of 30 female commercial piglets (24±6 h old, 1.46±0.04 kg initial body weight) were individually housed and assigned to 1 of 3 treatments based on their body weight and litter. Piglets received milk replacer diets that varied in their calcium and phosphorus concentrations: deficient (0.78% Ca and 0.60% P; CaPD), adequate (1.08% Ca and 0.84% P; CaPA), or excess (1.38% Ca and 1.08% P; CaPE). The calcium and phosphorus concentrations in CaPA were based on sow-milk composition and an extrapolation of the NRC requirement (NRC, 2012) for older pigs (Table 2.1). The ratio of calcium to phosphorus was kept constant at 1.3, and all other nutrients were consistent across the treatments. Dietary calcium and phosphorus concentrations in the milk replacers were altered with the addition of monobasic and dibasic calcium phosphate (Sigma). The liquid milk replacer was reconstituted with distilled and deionized water (175 g dry milk replacer

reconstituted to 1 kg of liquid milk replacer) and provided 8 times/d (08:00–23:00 h) through a gravity-flow liquid feed delivery system as described previously (Cooper et al., 2006) All piglets were limit-fed equal quantities of milk replacer designed to match the growth rate of sow-reared piglets. Piglets were on trial for 16 d. Four pigs with poor growth performance due to health issues unrelated to the dietary treatments were excluded from the study, which left a total of 26 pigs with 9 pigs in each of CaPD and CaPA groups and 8 pigs in the CaPE group. All pigs received bromodeoxyuridine (BrdU; Sigma) orally at 25 mg/kg body weight 15 h before tissue collection for in vivo MSC and SC proliferation evaluation.

SC Isolation

SC isolations from individual pigs were obtained (Alexander et al., 2012) and cultured as described previously (Chen et al., 2017)and were verified to be >95% Pax7+.Cryopreserved SCs were cultured in growth media (MEM+10%FBS+antibiotics) at 37◦C in a humidified environment containing 5% CO_2 in 15-cm plates coated with Matrigel matrix (Corning) with complete media changes every other day until 80% confluence. Cells were then trypsinized and replated at a density of 2×10^3 cells/cm^2 for evaluation of proliferation, and at a density of 2.5×10^4 cells/cm^2 for determination of differentiation.

SC Proliferation and Differentiation

For analysis of *in vitro* differentiation, SCs were cultured in differentiation media (MEM+2% horse serum+antibiotics) for 3 d. At the end of differentiation cells were subjected to immunocytochemical staining for the presence of myosin heavy chain. Briefly, cells were fixed with 4% paraformaldehyde for 15 min and washed twice with ice-cold PBS. The samples were then incubated for 10 min with PBS containing 0.25% Triton X-100 (PBST) and washed in PBS 3 times for 5 min. Cells were blocked in1% BSA in PBST, and incubated with mf20 antibody

(Developmental Studies Hybridoma Bank) overnight at 4°C. On the second day, samples were washed in PBS 3 times for 5 min, incubated with Dylight 488second antibody (Thermo Fisher Scientific) for 1 h, and washed in PBS 3 times for 5 min. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) in PBS. Pictures were taken at 20×magnification. The total number of nuclei were counted, and the fusion index was presented as a ratio of nuclei within myotubes compared with the total nuclei. *In vitro* SC proliferation was determined at 0, 24, 48, and 72 hours using the CyQUANT™ NF Cell Proliferation Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Gene Expression Analysis

Analysis of gene expression was performed as described previously (Rhoads et al., 2013). Briefly, total RNA was isolated from ~50 mg of *longissimus dorsi* tissue using TRIzol reagent (Invitrogen, Waltham, MA). Micro RNA was isolated from ~50 mg of *longissimus dorsi* tissue and satellite cells at 24 hours and 72 hours of differentiation using the mirVana™ miRNA Isolation Kit (Life Technologies, Carlsbad, CA) according to manufacturer's instructions. Quantity and quality of RNA was verified using the Experion System (Bio-Rad Laboratories, Hercules, CA). All RNA had an RNA integrity number above 8. The eluted miRNA was quantified by absorbance at 260 nm and 1,000ng of RNA and miRNA were reverse transcribed with iScript reverse transcriptase (Bio-Rad Laboratories, Hercules, CA). Micro RNA were reverse-transcribed using the stem loop method previously described by Kramer in 2011 by including a stem-loop primer in the reaction mix. Traditional PCR requires a template that is at least two times the length of the primers. Since miRNA's are 17-24 nucleotides in length, a stem loop primer is provided to bind to miRNA and increase their length to allow polymerase to bind and replicate. Real-time SYBR green PCR assays were performed with RNA and miRNA-specific primers (Tables 2.2 and 2.3).

PCR reactions were performed in a 20µl volume using SsoAdvanced universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). Reactions contained a 1-l primer set and diluted cDNA (10 ng). Each sample was quantified in triplicate. SYBR Green fluorescence was quantified with the CFX96 Touch Real Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). A total of 40 PCR cycles were run, and a dissociation curve was included to verify the amplification of a single PCR product. Muscle tissue relative mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method with the average of 18S and API5 abundance used for normalization. A standard curve was generated with oligonucleotides of the mature miRNAs measured to quantify miRNA abundance. All primer sets were evaluated for adequate efficiency before use in this study.

Statistical Analysis

The normality for all data was tested using the Proc Univariate procedure of SAS. Homogeneity of variance was evaluated by Levene's test. Body weight, feed efficiency, and bone parameters were analyzed by Proc glimmix procedure with dietary treatment as the main effect. Tukey's test was used for multiple comparison tests. Both in vivo and *in vitro* proliferations of SCs and MSCs were analyzed using the Proc Mixed procedure of SAS, with dietary treatment as the fixed effect for the in vivo analysis, and dietary treatment and time as fixed effects for the *in vitro* studies. The differentiation of SCs was analyzed using the Proc Mixed procedure of SAS, with dietary treatment as the fixed effect for in vivo studies, and dietary treatment and time as fixed effects for the *in vitro* analysis. Real time PCR data for total RNA were analyzed using the $2^{-\Delta\Delta CT}$ method after normalization with the geometric mean of TOP2B and API5 expression. Gene expression was normalized back to the adequate treatment group for tissue gene expression, and adequate treatment on 24 hours for satellite cell miRNA gene expression. The relative quantity ($2^{-\Delta\Delta CT}$) values were subjected to ANOVA analysis. Gene expression as analyzed by Proc mixed

procedure with different diet treatments as the main effect. Tukey's test was used for pairwise comparison if there is significant diet or time effects. Tukey's test was used for multiple means comparisons for all analyses. Differences were considered significant at $P<0.05$.

Results

Body Weight

At the completion of the study, piglets had reached 4.3 ±0.09 kg of body weight and body weight gain per kilogram of feed intake was 1.15±0.09. There was no significant effect of dietary treatment on body weight gain or feed efficiency (Zhang et al., 2020).

SC Proliferation and Differentiation

SCs isolated from CaPD-fed pigs had reduced in vivo proliferating SCs [BrdU/myoblast determination protein 1 (MyoD)+] by 19% ($P<0.05$) compared with those from CaPA- and CaPE-fed pigs, which were not statistically different from each other (Figure 2.1) (Zhang et al., 2020). However, *in vitro*, SCs isolated from pigs fed the CaPE diet had a 13% lower proliferation rate compared to those from CaPA-fed pigs ($P=0.08$) with those from the CaPD fed pigs being intermediate and not different from SCs from pigs fed the other diets (Figure 2.1). To evaluate *in vitro* differentiation, fusion rates were evaluated by comparing the number of nuclei within a myotube with total nuclei present. Myoblast fusion rates were greatest among cells isolated from CaPA-fed pigs, and cells isolated from CaPE-fed pigs exhibited 19% lower fusion rates than those from the CaPA-fed pigs ($P<0.05$) (Figure 2.2). SCs isolated from CaPD fed pigs had fusion rates that were not different from those of cells from CaPA-fed pigs but tended to be ~11% greater than those from CaPE-fed pigs ($P=0.08$) (Figure 2.2).

Gene Expression

Dietary inclusion level of CaP did not influence the expression of MyoD, MyoG, myostatin, IGF1 (Figure 2.3). The expression of Pax7 tended to differ between treatments (P=0.09) with CaPA pigs having 15% greater expression than CaPD pigs. CaPD pigs tended to have a 23% greater expression of Mef2a than CaPE pigs (P=0.09). There were no differences in SDH, AMPK, CS, or calcineurin expression (Figure 2.4). CaPD had a tendency (P=0.07) to have 29% greater expression of 4EBP1 than CaPE pigs. Myosin heavy chain expression did no differ between treatments for any isoform (Figure 2.5).

Micro-RNA

There were no differences between dietary treatments in any miRNA analyzed in tissue from the *longissimus dorsi* muscle (Figure 2.6). In response to differing CaP levels in the diet, miR-206 had a tendency to be 9% higher in differentiating satellite cells from CaPA pigs than CaPD and CaPE (P=0.07). There were no differences in miR-1, -133a, or -133b in differentiating satellite cells in response to diet or time (Figure 2.7).

Discussion

Although calcium and phosphorus nutrition are critical for both appropriate bone and soft tissue growth, their role in bone growth and development has received the greatest attention. We have also demonstrated that dietary phosphorus deficiency during neonatal development reduced body growth rate and feed conversion efficiency, as well as altering the in vivo proliferations of SCs (Alexander et al., 2010). In this study, we examined a dual deficiency of calcium and phosphorus in neonatal pigs. Our data support that even a marginal calcium and phosphorus deficiency impacts satellite cell function.

Early-life nutrient restriction reduces SC numbers and activity, as well as reduces overall muscle growth (Halevy et al., 2000). Reductions in SC numbers in early life cause lifetime deficits in muscle accretion (Fiorotto and Davis, 2018). Both calcium and phosphorus are necessary for muscle development, and deficiencies in either nutrient can cause a decrease in muscle growth due to altered SC function (Alexander et al., 2012; Tu et al., 2016). In both pigs and chickens, early-life phosphorus restriction has been shown to have a greater impact on carcass composition than phosphorus restriction in later life (Driver et al., 2006; Alexander et al., 2010; Alexander et al., 2012). In the current study, dietary deficiency of calcium and phosphorus resulted in a decrease in SC proliferation in vivo. However, our *in vitro* results indicate that SCs isolated from CaPE-fed pigs had compromised *in vitro* proliferation. In vitro proliferation rate in cells isolated from pigs fed the CaPD diet was slightly higher than those from the CaPE group but significantly lower than those from the CaPA-fed pigs. The ability of SCs to proliferate and progress through their myogenic lineage is key to the development and growth of neonatal muscle (Relaix and Zammit, 2012). While differing results occurred *in vitro* versus in vivo, alterations in calcium and phosphorus in neonatal diets alters SC activity. Alterations in SC activity can impact the lean growth potential of animals. Our data indicate that dietary calcium and phosphorus deficiency reduces SC proliferation and progression through their myogenic lineage, which will lead to a reduction in muscle growth.

Satellite cells are characterized by Pax7 expression. During quiescence, Pax7 functions to maintain the growth-arrested state and inhibit differentiation, and its expression is high. Once the satellite cell is activated its expression remains high throughout proliferation, and is downregulated as satellite cells progress through the myogenic lineage (Buckingham, 2007; Kuang et al., 2007). In this study, CaPA pigs had higher expression of Pax7 in tissue which could indicate a greater

number of satellite cells. This coincides with satellite cells from the CaPA treatment having the highest proliferation rate (Zhang et al., 2020).

Progression of satellite cells through the myogenic lineage is accomplished through the precise expression patterns of myogenic genes. During the process of stem cell commitment to the myogenic lineage, miRNAs function to facilitate and refine the complex expression pattern of myogenic genes through inhibition of translation or marking target mRNAs for degradation. Micro-RNA -1 and -206 differ from one another by one nucleotide that resides outside of the seed sequence, therefore the two miRNAs share many of the same targets (Dey et al., 2011). Differentiation is promoted by miR-1/206 through targeting histone deacetylase 4 (HDAC4), a transcriptional repressor of myogenic genes mainly inhibiting the transcription factor MEF2A and-C, which are important in differentiation and muscle hypertrophy (Lu et al., 2000; Ikeda et al., 2009). In this study Mef2a had the highest expression in tissue in CaPD diets over CaPE. This corresponds with CaPD diets also having higher fusion index than CaPE diets. The concentration of miR-206 in satellite cells tended to be higher in CaPA treatment which parallels the highest fusion index in CaPA pigs (Zhang et al., 2020).

Conclusion

The data from this study provide strong support for the importance of neonatal calcium and phosphorus nutrition for muscle development. Even a relatively minor deficiency in calcium and phosphorus altered the function of the stem cells responsible for muscle growth. Alterations in the function of tissue-specific stem cells that play important roles in regulating lifetime lean growth potential highlight the importance of neonatal nutrition and the need to further our understanding of the long-term effects of calcium and phosphorus nutrition in neonates.

Acknowledgments

This study was funded in part by USDA (2015-67015-25094).

A portion of this paper was previously published in: Wei Zhang, Kellie A Kroscher, Robert L Murray, Rachel Gagliardi, Carly Guiltinan, Robert P Rhoads, Chad H Stahl, Dietary Calcium and Phosphorus Amounts Affect Development and Tissue-Specific Stem Cell Characteristics in Neonatal Pigs, *The Journal of Nutrition*, Volume 150, Issue 5, May 2020, Pages 1086–1092, https://doi.org/10.1093/jn/nxaa011

References

Alexander, L. S., A. Mahajan, J. Odle, K. L. Flann, R. P. Rhoads, and C. H. Stahl. 2010. Dietary Phosphate Restriction Decreases Stem Cell Proliferation and Subsequent Growth Potential in Neonatal Pigs. The Journal of Nutrition 140(3):477-482. doi: 10.3945/jn.109.117390

Alexander, L. S., B. S. Seabolt, R. P. Rhoads, and C. H. Stahl. 2012. Neonatal phosphate nutrition alters in vivo and in vitro satellite cell activity in pigs. Nutrients 4(6):436-448. doi: 10.3390/nu4060436

Bertram, M. J. 1995. The impact of dietary phosphorus regimen on muscle growth and quality in pig differing in lean growth capacity.

Buckingham, M. 2007. Skeletal muscle progenitor cells and the role of Pax genes. Comptes rendus biologies 330(6-7):530-533.

Chen, Y., H. Zhu, S. R. McCauley, L. Zhao, S. E. Johnson, R. P. Rhoads, and S. W. El-Kadi. 2017. Diminished satellite cell fusion and S6K1 expression in myotubes derived from skeletal muscle of low birth weight neonatal pigs. Physiological Reports 5(3):e13075. doi: 10.14814/phy2.13075

Dey, B. K., J. Gagan, and A. Dutta. 2011. miR-206 and-486 induce myoblast differentiation by downregulating Pax7. Molecular and cellular biology 31(1):203-214.

Driver, J. P., G. M. Pesti, R. I. Bakalli, and H. M. Edwards. 2006. The Effect of Feeding Calcium- and Phosphorus-Deficient Diets to Broiler Chickens During the Starting and Growing-Finishing Phases on Carcass Quality. Poultry Science 85(11):1939-1946. doi: 10.1093/ps/85.11.1939

Fiorotto, M. L., and T. A. Davis. 2018. Critical windows for the programming effects of early-life nutrition on skeletal muscle mass, Recent Research in Nutrition and Growth No. 89. Karger Publishers. p. 25-35.

Halevy, O., A. Geyra, M. Barak, Z. Uni, and D. Sklan. 2000. Early Posthatch Starvation Decreases Satellite Cell Proliferation and Skeletal Muscle Growth in Chicks. The Journal of Nutrition 130(4):858-864. doi: 10.1093/jn/130.4.858

Ikeda, S., A. He, S. W. Kong, J. Lu, R. Bejar, N. Bodyak, K.-H. Lee, Q. Ma, P. M. Kang, T. R. Golub, and W. T. Pu. 2009. MicroRNA-1 negatively regulates expression of the hypertrophy-associated calmodulin and Mef2a genes. Molecular and cellular biology 29(8):2193-2204. doi: 10.1128/MCB.01222-08

Ji, Y., Z. Wu, Z. Dai, X. Wang, J. Li, B. Wang, and G. Wu. 2017. Fetal and neonatal programming of postnatal growth and feed efficiency in swine. Journal of Animal Science and Biotechnology 8(1):42. doi: 10.1186/s40104-017-0173-5

Kramer, M. F. 2011. Stem-loop RT-qPCR for miRNAs. Current protocols in molecular biology 95(1):15.10. 11-15.10. 15. doi: 10.1002/0471142727.mb1510s95

Kuang, S., K. Kuroda, F. Le Grand, and M. A. Rudnicki. 2007. Asymmetric Self-Renewal and Commitment of Satellite Stem Cells in Muscle. Cell 129(5):999-1010. doi: 10.1016/j.cell.2007.03.044

Lu, J., T. A. McKinsey, C.-L. Zhang, and E. N. Olson. 2000. Regulation of skeletal myogenesis by association of the MEF2 transcription factor with class II histone deacetylases. Molecular cell 6(2):233-244.

Relaix, F., and P. S. Zammit. 2012. Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage. Development 139(16):2845. doi: 10.1242/dev.069088

Rhoads, R., K. Flann, T. Cardinal, C. Rathbone, X. Liu, and R. E. Allen. 2013. Satellite cells isolated from aged or dystrophic muscle exhibit a reduced capacity to promote angiogenesis in vitro. Biochemical and biophysical research communications 440(3):399-404. doi: 10.1016/j.bbrc.2013.09.085

Shelton, J. L., L. L. Southern, F. M. LeMieux, T. D. Bidner, and T. G. Page. 2004. Effects of microbial phytase, low calcium and phosphorus, and removing the dietary trace mineral premix on carcass traits, pork quality, plasma metabolites, and tissue mineral content in growing-finishing pigs12. Journal of Animal Science 82(9):2630-2639. doi: 10.2527/2004.8292630x

Sugiura, S., A. Higashitani, and T. Sasaki. 2011. Effects of dietary phosphorus restriction on fillet fat deposition and hepatic lipid metabolism in rainbow trout (Oncorhynchus mykiss) and crucian carp (Carassius auratus grandoculis). Aquaculture Science 59(1):109-122. doi: 10.11233/aquaculturesci.59.109

Tu, M. K., J. B. Levin, A. M. Hamilton, and L. N. Borodinsky. 2016. Calcium signaling in skeletal muscle development, maintenance and regeneration. Cell Calcium 59(2):91-97. doi: https://doi.org/10.1016/j.ceca.2016.02.005

Zhang, W., K. A. Kroscher, R. L. Murray, R. Gagliardi, C. Guiltinan, R. P. Rhoads, and C. H. Stahl. 2020. Dietary Calcium and Phosphorus Amounts Affect Development and Tissue-Specific Stem Cell Characteristics in Neonatal Pigs. The Journal of Nutrition 150(5):1086-1092. doi: 10.1093/jn/nxaa011

Zimmerman, D., V. Speer, D. Catron, and V. Hays. 1960. Calcium studies with baby pigs. Journal of Animal Science 19(4):1301-1302.

Tables and Figures

Table 2.1 Composition of the CaPD diet.

Ingredient	%
Whey	52.7
Whey Protein Concentrate	19.9
11/45 MR High Fat Blend[1]	24.3
Limestone	0.05
Dicalcium Phosphate	0.03
Mineral Premix[2]	1.28
Vitamin Premix[3]	0.11
Lysine	0.11
Methionine	0.08
Citric Acid	1.00
Flavor	0.01
Lecithin Blend	0.43
Composition, calculated	
Crude Protein	25.0
Crude Fat	13.0
Ca	0.78
P	0.60

[1]Provided by Milk Specialties, Eden Prairie, MN.
[2]Mineral Premix was 7.275% Ca, 0.215% P, 0.111% Na, 12.309% Cl, 9.970% K, and provided 725.5 mg Cu, 9.216 g Zn, 23.53 mg Se, 78.43 mg Co, 0.157g I, 7.84 g Fe, and 1.961 g Mn per kg of premix.
[3]Vitamin Premix provides 22,046 IU Vitamin A, 4,409 IU Cholecalciferol, 110 IU Vitamin E per gram of premix. It also provides 3.41 g Vitamin K, 1.36 g Thiamine, 5.59 g Riboflavin, 2.66 g Pyridoxine, 29.33 mg Vitamin B12, 19.99 g Pantothenic acid, 22.05 g Niacin, 1.84 g Folic acid, 78 g Vitamin C, and 44 mg Biotin per Kg of premix.

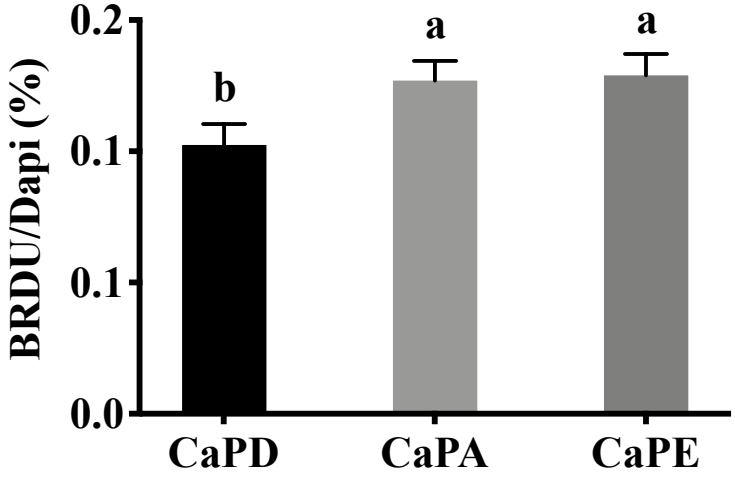
Table 2.2 Nucleotide sequences for real-time PCR primers

Target	Direction	Sequence
18S	Forward	GTAACCCGTTGAACCCCAT
	Reverse	CCA TCC AAT CGG TAG TAG CG
API5	Forward	GCATTTTTAGTAGCATAGGCCCTTT
	Reverse	AACTTGAGGGAAGATTAACTGTGGAA
AMPKinase	Forward	ACGGAGCAAGTAACCCCAGC
	Reverse	GCCACGAGGATGACGATGAA
IGF-1	Forward	GCACATCACATCCTCTTCGC
	Reverse	GCCTCCTCAGATCACAGCTC
MEF2A	Forward	TGAATACCCAGAGGATAAGCAGTT
	Reverse	TAATCGGTGTTGTAGGCGG
MyHC I	Forward	AAGGGCTTGAACGAGGAGTAGA
	Reverse	TTATTCTGCTTCCTCCAAAGGG
MyHC IIa	Forward	GCTGAGCGAGCTGAAATCC
	Reverse	ACTGAGACACCAGAGCTTCT
MyHC IIb	Forward	ATGAAGAGGAACCACATTA
	Reverse	TTATTGCCTCAGTAGCTTG
MyHC IIx	Forward	AGAAGATCAACTGAGTGAACT
	Reverse	AGAGCTGAGAAACTAACGTG
MSTN	Forward	CCAGAGAGATGACAGCAGTGATG
	Reverse	TTCCTTCCACTTGCATTAGAAGATC
MyoD	Forward	GCGTGCAAACGCAAGACCACTAA
	Reverse	AGTCTCGAAGGCCTCGTTGACTTT
MyoG	Forward	TGACCCTACAGATGCCCACAATCT
	Reverse	GTTGGGCATGGTTTCATCTGGGAA
Pax7	Forward	CAACCACATCCGCCACAAGATAGT
	Reverse	AGAGGATCTTGGAGACACAGCCAT
SDH	Forward	TGGCCGGCACAGCTCCCAAT
	Reverse	GCACGTTCCCTGCTCTCCCG

Table 2.3 Nucleotide sequences for miRNA real-time PCR primers

Target	Primer	Primer sequences (5'-3')
ssc-miR-1	Stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTACATA
	Forward	GGCGGCTGGAATGTAAAGAAG
	Reverse	CAGTGCAGGGTCCGAGGTAT
	Oligonucleotide	TGGAATGTAAAGAAGTATGTA
ssc-miR-133a-3p	Stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGCTG
	Forward	TTGCGTTGGTCCCCTTCA
	Reverse	CAGTGCAGGGTCCGAGGTAT
	Oligonucleotide	TTGGTCCCCTTCAACCAGCTG
ssc-miR-133b	Stem-loop	GCGTGGTCCACACCACCTGAGCCGCCACGACCACGCATAGCTGG
	Forward	CCAGCCTTTGGTCCCCTTC
	Reverse	TCCACACCACCTGAGCCG
	Oligonucleotide	TTTGGTCCCCTTCAACCAGCTAT
ssc-miR-206	Stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCACAC
	Forward	CCGCGTGGAATGTAAGGAAGT
	Reverse	CAGTGCAGGGTCCGAGGTAT
	Oligonucleotide	TGGAATGTAAGGAAGTGTGTGA

a



b

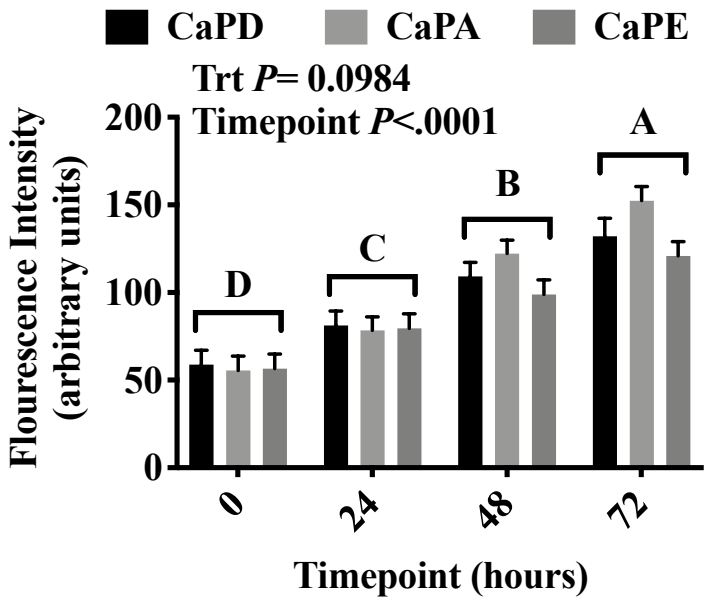


Figure 2.1 Effects of dietary inclusion level of $CaPO_4$ on satellite cell proliferation. A) Percentage of BrdU positive cells from the longmissus dorsi muscle of neonatal pigs fed a 25% deficient Calcium Phosphate (CaPD), adequate Calcium Phosphate (CaPA), or a 25% excess Calcium Phosphate (CaPE) diet. Results are means ±SEM. Treatments without a common letter are different at $P \leq 0.05$. B) Proliferation rate of satellite cells from neonatal pigs fed CaPD, CaPA, or a CaPE diet at 0, 24, 48 and 72 hours. Results are means ±SEM. Timepoints without a common uppercase letter are different at $P \leq 0.05$. Means without a common lower-case letter are different among treatments within each timepoint at $P \leq 0.05$. (Previously published (Zhang et al., 2020)

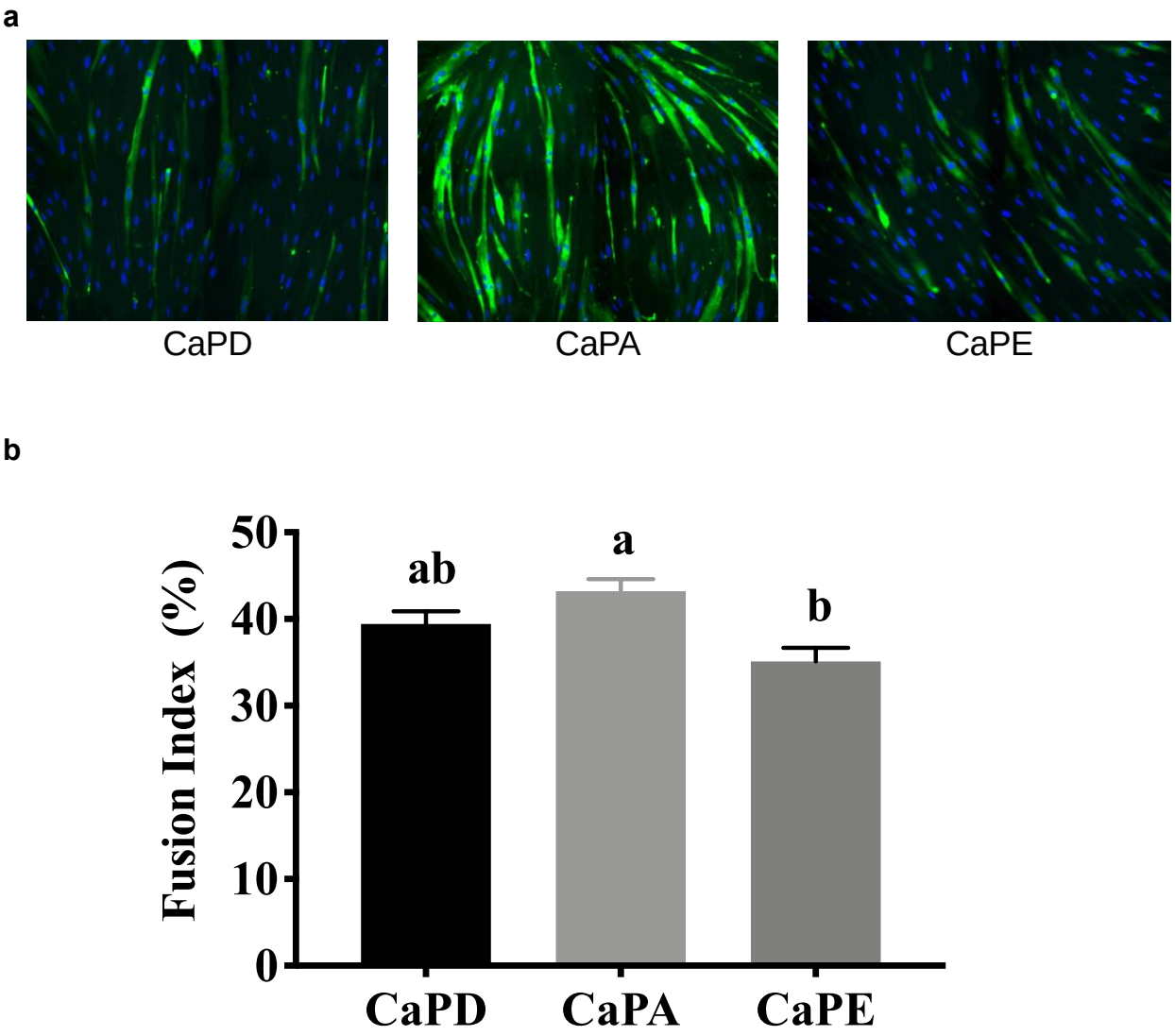


Figure 2.2 The effect of $CaPO_4$ inclusion levels on satellite cell differentiation. A) Representative images of myogenic differentiation of satellite cells from neonatal pigs fed a 25% deficient Calcium Phosphate (CaPD), adequate Calcium Phosphate (CaPA), or a 25% excess Calcium Phosphate (CaPE) diet. Immuno-labelling in satellite cell-derived myotubes at day 3 of differentiation. Original to the manuscript. B) Fusion index expressed as the percentage of total nuclei that are located in myotubes of satellite cells of pigs fed a diet containing either a CaPD, CaPA, or CaPE diet. Values are means ± SEMs. Means at a time without a common letter differ $P < 0.05$.

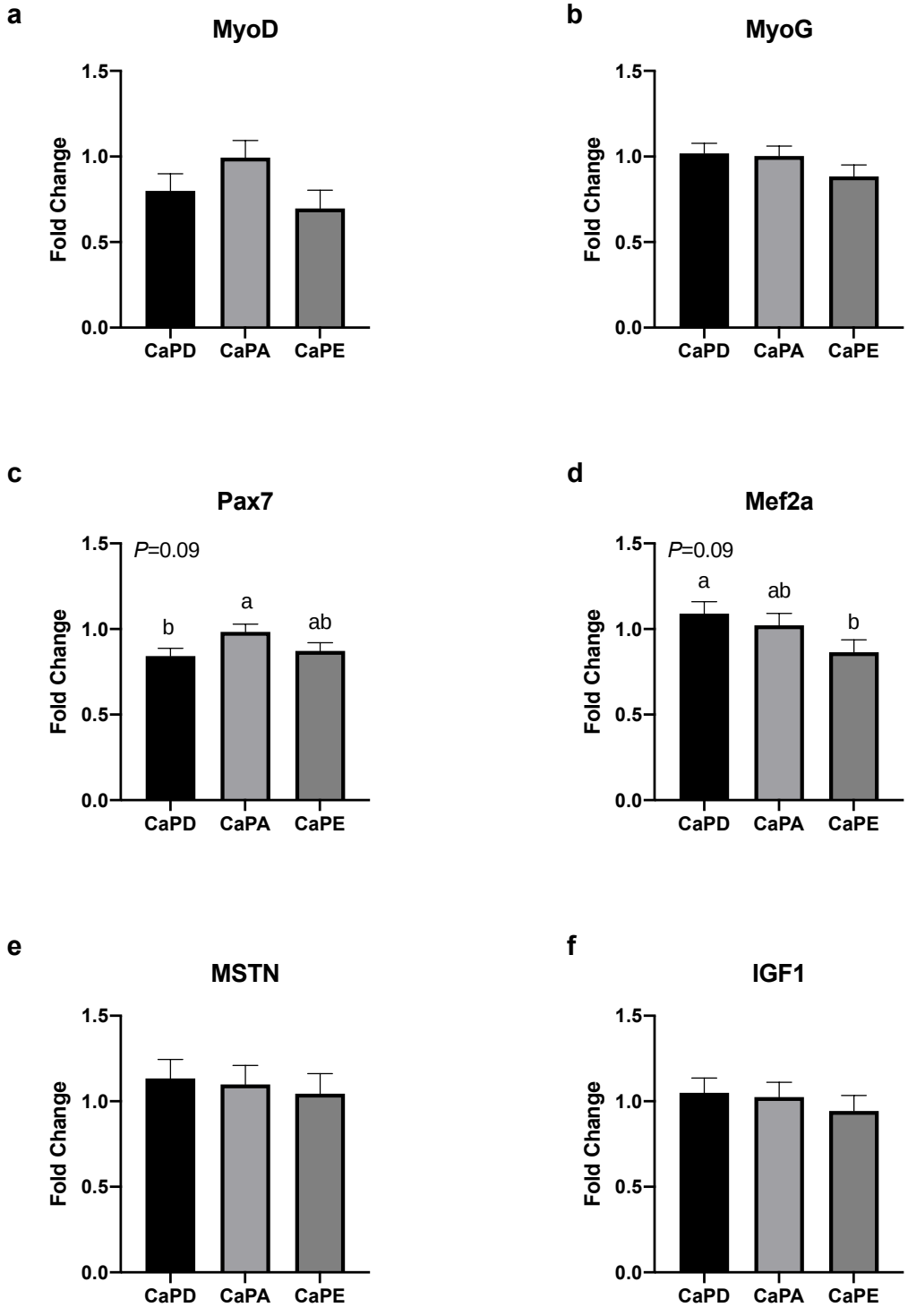


Figure 2.3 The effect of $CaPO_4$ inclusion levels on mRNA expression of genes involved in muscle growth in the *longissimus dorsi* muscle. Pigs were fed a diet containing either 25% deficient (CaPD), (CaPA), or a 25% excess Calcium Phosphate (CaPE) diet. Results are means ± SEM. Means without a common letter are different at $P<0.05$ (a>b).

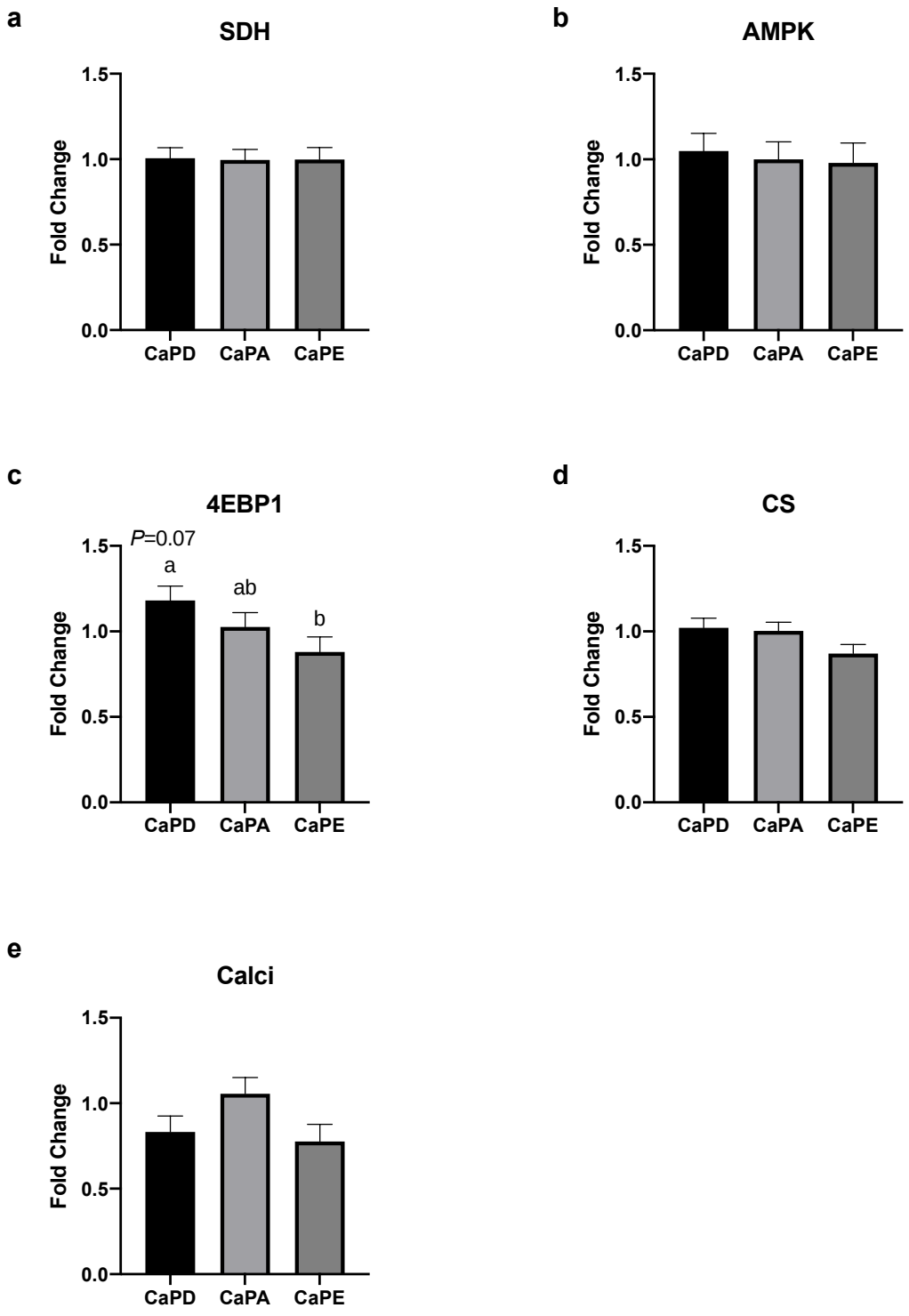


Figure 2.4 The effects of dietary $CaPO_4$ on mRNA expression of genes involved in energy sensing in the *longissimus dorsi* muscle. Pigs were fed a diet containing either 25% deficient (CaPD), adequate (CaPA), or a 25% excess $CaPO_4$ (CaPE) diet. Results are means ± SEM. Means without a common letter are different at $P<0.05$ (a>b).

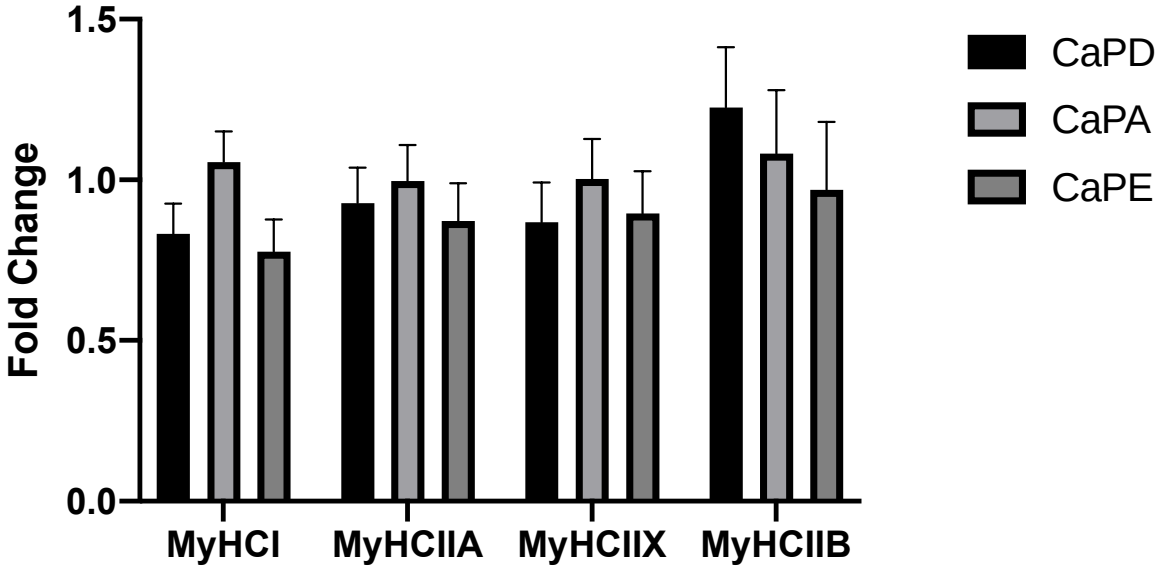


Figure 2.5 mRNA expression of myosin heavy chain (MyHC) isoforms in the *longissimus dorsi* muscle of pigs fed a diet containing either 25% deficient Calcium Phosphate (CaPD), adequate Calcium Phosphate (CaPA), or a 25% excess Calcium Phosphate (CaPE) diet. Results are means ± SEM. Means were considered different at $P<0.05$.

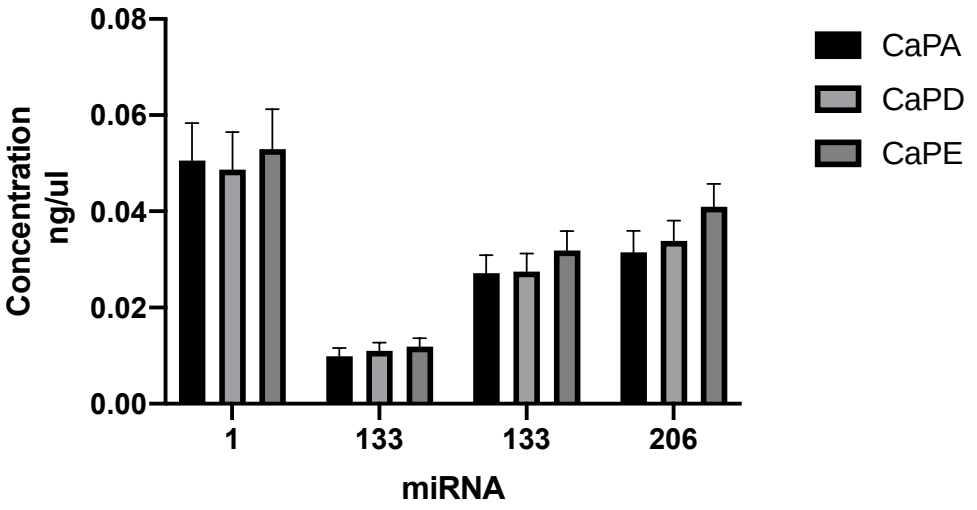


Figure 2.6 Expression of miR-1, miR-133a, miR-133b, and miR-206 in *longissimus dorsi* muscle of pigs fed differing levels of $CaPO_4$. Pigs were fed a diet containing either 25% deficient (CaPD), adequate (CaPA), or a 25% excess Calcium Phosphate (CaPE) diet. Results are means ± SEM. Means were considered different at $P<0.05$.

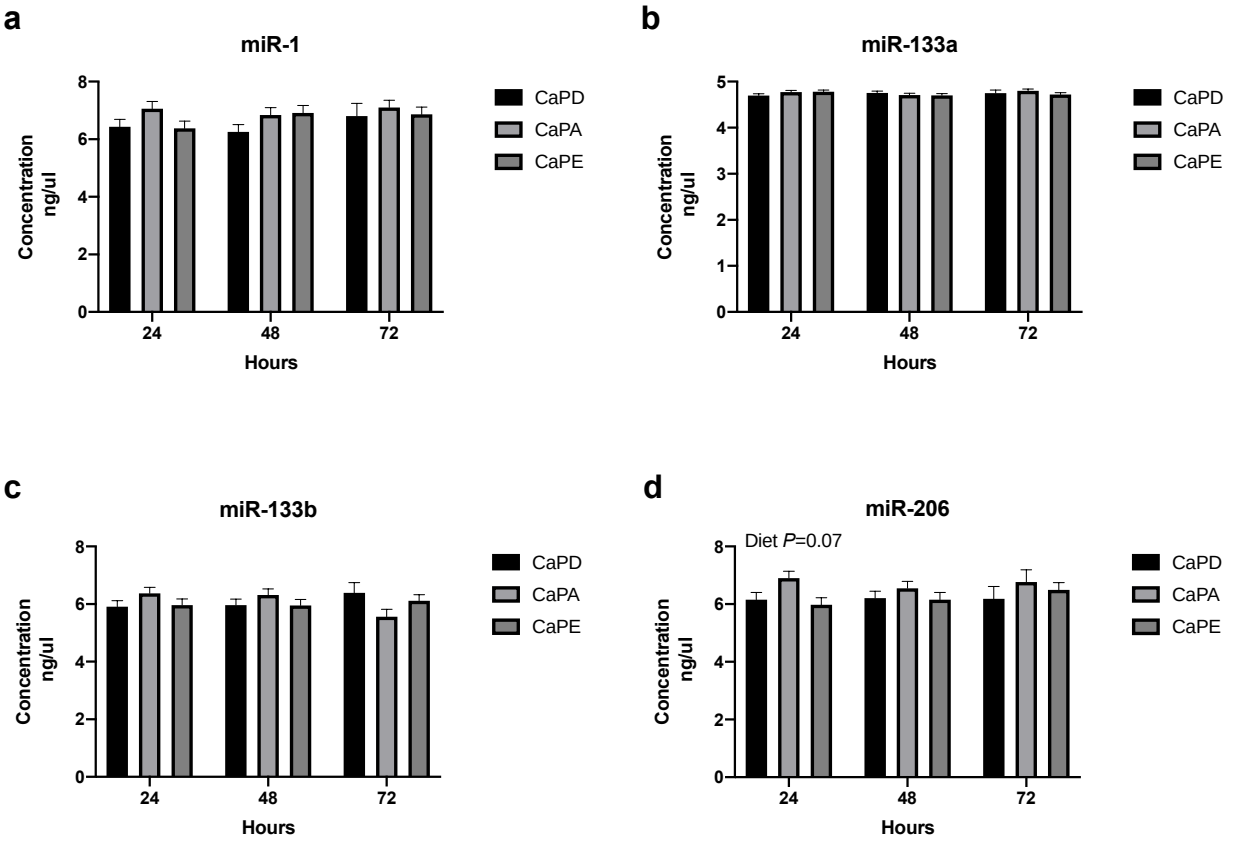


Figure 2.7 Expression of miR-1 (A), miR-133a (B), miR-133b (C), and miR-206 in satellite cells of pigs fed differing levels of $CaPO_4$. Pigs were fed a diet containing either 25% deficient Calcium Phosphate (CaPD), adequate Calcium Phosphate (CaPA), or a 25% excess Calcium Phosphate (CaPE) diet at 0 hours and 72 hours of differentiation. Results are means ± SEM. Means without a common letter are different at $P<0.05$.

References

Alexander, L. S., A. Mahajan, J. Odle, K. L. Flann, R. P. Rhoads, and C. H. Stahl. 2010. Dietary Phosphate Restriction Decreases Stem Cell Proliferation and Subsequent Growth Potential in Neonatal Pigs. The Journal of Nutrition 140(3):477-482. doi: 10.3945/jn.109.117390

Alexander, L. S., B. S. Seabolt, R. P. Rhoads, and C. H. Stahl. 2012. Neonatal phosphate nutrition alters in vivo and in vitro satellite cell activity in pigs. Nutrients 4(6):436-448. doi: 10.3390/nu4060436

Bertram, M. J. 1995. The impact of dietary phosphorus regimen on muscle growth and quality in pig differing in lean growth capacity.

Buckingham, M. 2007. Skeletal muscle progenitor cells and the role of Pax genes. Comptes rendus biologies 330(6-7):530-533.

Chen, Y., H. Zhu, S. R. McCauley, L. Zhao, S. E. Johnson, R. P. Rhoads, and S. W. El-Kadi. 2017. Diminished satellite cell fusion and S6K1 expression in myotubes derived from skeletal muscle of low birth weight neonatal pigs. Physiological Reports 5(3):e13075. doi: 10.14814/phy2.13075

Cooper, C., S. Westlake, N. Harvey, K. Javaid, E. Dennison, and M. Hanson. 2006. Review: developmental origins of osteoporotic fracture. Osteoporos Int 17(3):337-347. doi: 10.1007/s00198-005-2039-5

Dey, B. K., J. Gagan, and A. Dutta. 2011. miR-206 and-486 induce myoblast differentiation by downregulating Pax7. Molecular and cellular biology 31(1):203-214.

Driver, J. P., G. M. Pesti, R. I. Bakalli, and H. M. Edwards. 2006. The Effect of Feeding Calcium- and Phosphorus-Deficient Diets to Broiler Chickens During the Starting and Growing-Finishing Phases on Carcass Quality. Poultry Science 85(11):1939-1946. doi: 10.1093/ps/85.11.1939

Fiorotto, M. L., and T. A. Davis. 2018. Critical windows for the programming effects of early-life nutrition on skeletal muscle mass, Recent Research in Nutrition and Growth No. 89. Karger Publishers. p. 25-35.

Halevy, O., A. Geyra, M. Barak, Z. Uni, and D. Sklan. 2000. Early Posthatch Starvation Decreases Satellite Cell Proliferation and Skeletal Muscle Growth in Chicks. The Journal of Nutrition 130(4):858-864. doi: 10.1093/jn/130.4.858

Ikeda, S., A. He, S. W. Kong, J. Lu, R. Bejar, N. Bodyak, K.-H. Lee, Q. Ma, P. M. Kang, T. R. Golub, and W. T. Pu. 2009. MicroRNA-1 negatively regulates expression of the hypertrophy-associated calmodulin and Mef2a genes. Molecular and cellular biology 29(8):2193-2204. doi: 10.1128/MCB.01222-08

Ji, Y., Z. Wu, Z. Dai, X. Wang, J. Li, B. Wang, and G. Wu. 2017. Fetal and neonatal programming of postnatal growth and feed efficiency in swine. Journal of Animal Science and Biotechnology 8(1):42. doi: 10.1186/s40104-017-0173-5

Kramer, M. F. 2011. Stem-loop RT-qPCR for miRNAs. Current protocols in molecular biology 95(1):15.10. 11-15.10. 15. doi: 10.1002/0471142727.mb1510s95

Kuang, S., K. Kuroda, F. Le Grand, and M. A. Rudnicki. 2007. Asymmetric Self-Renewal and Commitment of Satellite Stem Cells in Muscle. Cell 129(5):999-1010. doi: 10.1016/j.cell.2007.03.044

Lu, J., T. A. McKinsey, C.-L. Zhang, and E. N. Olson. 2000. Regulation of skeletal myogenesis by association of the MEF2 transcription factor with class II histone deacetylases. Molecular cell 6(2):233-244.

NRC, N. R. C. 2012. Nutrient requirements of swine. National Academies Press.

Relaix, F., and P. S. Zammit. 2012. Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage. Development 139(16):2845. doi: 10.1242/dev.069088

Rhoads, R., K. Flann, T. Cardinal, C. Rathbone, X. Liu, and R. E. Allen. 2013. Satellite cells isolated from aged or dystrophic muscle exhibit a reduced capacity to promote angiogenesis in vitro. Biochemical and biophysical research communications 440(3):399-404. doi: 10.1016/j.bbrc.2013.09.085

Shelton, J. L., L. L. Southern, F. M. LeMieux, T. D. Bidner, and T. G. Page. 2004. Effects of microbial phytase, low calcium and phosphorus, and removing the dietary trace mineral premix on carcass traits, pork quality, plasma metabolites, and tissue mineral content in growing-finishing pigs12. Journal of Animal Science 82(9):2630-2639. doi: 10.2527/2004.8292630x

Sugiura, S., A. Higashitani, and T. Sasaki. 2011. Effects of dietary phosphorus restriction on fillet fat deposition and hepatic lipid metabolism in rainbow trout (Oncorhynchus mykiss) and crucian carp (Carassius auratus grandoculis). Aquaculture Science 59(1):109-122. doi: 10.11233/aquaculturesci.59.109

Tu, M. K., J. B. Levin, A. M. Hamilton, and L. N. Borodinsky. 2016. Calcium signaling in skeletal muscle development, maintenance and regeneration. Cell Calcium 59(2):91-97. doi: 10.1016/j.ceca.2016.02.005

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Zimmerman, D., V. Speer, D. Catron, and V. Hays. 1960. Calcium studies with baby pigs. Journal of Animal Science 19(4):1301-1302.

Chapter 3: Dietary Protein Inclusion Levels Enhances Neonatal Pig Growth and Alters Satellite Cell Activity

Abstract

Nutrition during neonatal life impacts developmental growth programing by altering SC activity. Growth as a percentage of body weight is highest during the neonatal period and restricting neonatal nutrition can lead to permanent muscle growth deficits. Despite this, our knowledge of the nutritional requirements needed to support this rapid growth is limited. The heightened responsiveness of protein synthesis during the neonatal period is mediated mainly by the activation of translation initiation factors. Post-transcriptional regulation of RNA is mediated in part by micro-RNAs (miRNA) that prevent translation and/or promote degradation and are known to be important modulators of stem cell activity. The objective of this study was to determine the impact of a protein deficient, adequate, and excess diet on muscle growth. Twenty-eight piglets obtained at 1d of age received either an adequate protein diet (A), a 25% deficient diet (D), or a 25% excess protein (E) diet over a 17-day trial. Piglet starting weights were not different however, final body weight and ADG were increased in excess pigs than deficient (5.60±0.27 kg vs 4.75±0.27 kg and 0.27±0.01 kg/d vs 0.21±0.01kg/d) despite an equal feed intake across treatments. Histological analysis indicated that *longissmus dorsi* (LD) muscle had significantly greater (P=0.02) muscle cross-sectional area and fiber cross-sectional area in excess than deficient treatment (muscle CSA E: 6.53±0.31 cm^2; D: 5.23±0.31; Fiber CSA E: 691.05±67.99 μm^2; D: 462.07±28.36 μm^2). Gene expression in the LD was evaluated by qPCR. There were no differences in myogenic genes MyoD and MyoG between treatments; however, IGF1 was lower in excess pigs than adequate and deficient pigs (P=0.006; D: 1.1649±0.08; A: 1.02±0.08, E: 0.76±0.09) despite the increased growth rates of excess treatment pigs. However, oxidative capacity marker SDH tended to be greater in excess pigs than deficient and excess (P=0.076) (D: 1.05±0.16; A: 1.04±0.16, E: 1.58±0.018). There were no differences in degradation

pathway genes muRF-1 or atrogin-1, but FOXO was increased in deficient pigs (*P*=0.016; D: 1.15±0.07; A: 1.02±0.07, E: 0.82±0.008) which may contribute to the reduced growth rate of deficient pigs. Energy sensing AMPK was decreased in deficient pigs (*P*=0.041; D: 0.7164±0.0.11; A: 1.04±0.11, E: 1.1196±0.012). The translational repressor 4EBP1 was upregulated in deficient pigs (*P*=0.005; D: 1.304±0.0.08; A: 1.03±0.08, E: 0.85±0.09). The differences in growth response may be due to post-transcriptional regulation; however, no differences were observed in miRNA expression in LD muscle tissue. The differentiation promoting miRs-1, 133b and 206 and proliferation promoting miR-133a were not different. While miRNA expression was not different between treatments in muscle tissue, miRNAs may still play a role in the differences observed in growth between deficient and excess treatments through their activity and rate of binding to their target mRNAs. Satellite cell proliferation was not different between treatments. As expected, satellite cell proliferation tended to increase over time (*P*=0.08). A diet by time interaction (P<0.01) was noted for myoblast fusion rates in which all treatments at day three had a lower myoblast fusion index than day 0 adequate and deficient diets. At day 3 of differentiation, deficient treatment had the lowest in myoblast fusion rates compared to adequate and excess treatments (*P*<0.01). Diet had a tendency to be interactively influenced by time (*P*=0.06) in which differentiating satellite cells in excess pigs at day 0 had higher levels of expression of differentiation promoting miR-1, and adequate pigs at day 3 had the lowest expression compared to other diets at each timepoint. Expression of miR-133a, -133b, and 206 were not different between diets in differentiating satellite cells. These findings suggested that dietary protein levels in excess of what is currently considered required during the neonatal period allows for improved growth and feed efficiency. Additionally, early dietary protein deficiency delays muscle growth.

Introduction

Animal fractional growth rate and body protein deposition are at its highest during neonatal period. Skeletal muscle growth and gain in protein mass occurs at a faster rate than other tissues during this period with high fractional rates of protein synthesis (Davis et al., 1989; Davis et al., 1996). Dietary amino acids utilization is highly efficient in the neonate and decline with ages likely due to an enhanced stimulation response to feeding during this period (McCracken et al., 1980; Davis et al., 1996; Davis et al., 1997). Muscle growth is driven by protein accretion, myonuclear incorporation, and accumulation of extracellular matrix (Allen et al., 1979; Laurent et al., 1985; Mozdziak et al., 1997). Satellite cells provide nuclei for incorporation into myofibers to facilitate the increased demand for protein production during hypertrophy. Remaining quiescent until activated, satellite cells proliferate, differentiate, and fuse to form new fibers or join with existing fibers (Miersch et al., 2017). In order to maintain the satellite cell pool, they are also capable of self-renewal. In order to undergo these changes, genes must be highly coordinated. Non-coding RNAs like micro RNA (miRNA) are highly involved in the myogenic process and function to fine-tune gene expression through translation inhibition and/or degradation (Hamilton and Baulcombe, 1999; Reinhart et al., 2000; Krol et al., 2004)

Skeletal muscle constitutes a large proportion of body mass and muscle growth attributes to a majority part of the mass increase, which is sensitive to dietary crude protein level. It has been well established that neonatal nutrition exerts a life-long impact on growth rate, bone development, and overall health later in life. Dietary protein can be metabolized in the stomach and small intestine, and a majority of amino acids (AA) are absorbed by the duodenum and jejunum, which constitute AA pools in the body. Animal growth could be directly stimulated by either the increases in AA pool, or indirectly enhanced by hormonal secretion such as insulin and growth hormone

(Davis and Fiorotto, 2009). Serum urea nitrogen level is a good indicator for the protein status and reflects protein metabolism and utilization.

Protein is an expensive resource for animal husbandry, and excess dietary crude protein could exceed the capacity of the hepatic and renal system to metabolism. As a result, excess nitrogen is excreted causing environment concerns. However, protein deficiency has adverse effects on animal growth. Previous studies showed that 6% unit reduction of protein in a 50-day trial decreased average daily gain and feed: grain ratio and muscle growth growing-finishing pigs (Li et al., 2016). Due to the lack of evidence in ideal protein supplementation level in neonatal pigs, this study sought to understand how different dietary protein levels alter animal growth through the regulation of muscle growth.

Methods

Animals

All pig protocols were approved by University of Maryland Animal Care and Use Committee. 30 1-day-old female commercial pigs (24±6h old, 1.47±0.1 kg initial body weight) were individually caged and assigned to 3 groups based on their body weight and genetic information. All pigs received an isocaloric diet with different protein levels. Dietary protein levels were adjusted by mixing milk replacer with different levels of whey protein supplementation to prepare feed that is either 25% deficient (19%) adequate (26%), or 25% excess (33%), in protein requirement. The energy level was adjusted by administering different levels of PEF, and lactose. Protein level in the adequate group was based on sow milk composition and an extrapolation of the NRC requirement for older pigs (NRC, 2012). Liquid weaning diet was provided 8 times a day from 8am to 11pm by reconstituting 145g of low protein milk replacer with different level of whey protein isolate, PEF, and lactose supplementation in 1 kg of water. The amount of feed provided

daily was designed to mimic the normal growth rate under sow-reared conditions. Pigs were grown to 17 days of age. Intravenous blood was collected at day 11 and day 17 for serum IGF-1 metabolite analyses. Pigs were euthanized using penetrating captive bolt gun followed by complete exsanguination. Longissimus dorsi (LD) muscle was collected for satellite cell isolation, gene expression, protein expression, and cross-sectional area (CSA) measurements.

Serum Hormone Concentrations

Serum concentrations of hormones related to protein and energy metabolism were measured. For instance, serum urea nitrogen, phosphate concentration, and IGF1 (LSBio, LS-F23280) were measured at both d11 and d17 according to the manual instructions. Urea is a main waste product of protein catabolism. To determine serum urea nitrogen concentration, a series of urea nitrogen standards was prepared at the concentration of 0, 0.25, 0.5, 1, 2, 3, 5, 7 mg/100 ml. Phenolphthalein indicator was prepared by adding 10g phenolphthalein in 600 mL of methanol and then adding 400 ml of water. Diacetylmonoxime reagent was prepared by dissolving 10 g of Arsenic Pentoxide and 7g of NaOH in 800mL of DI H_2O. After the solution was dissolved, 2 drops of phenolphthalein indicator were added to turn the solution into pink. Concentrated H_2SO_4 was then added until the pink color just disappears following additional 28 mL of concentrated H_2SO_4. When the solution was cooled to room temperature, 3 g of antipyrine phenazone, 5g of diacetylmonoxime, and additional H_2O were added to bring the solution up to 1L. Serum samples were then diluted 10-fold. Ten ul of sample dilutions and standards were mixed with 990 ul of diacetylmonoxime reagent respectively and heated at 100 degree for 15 min. After cooling, absorbance was measured at 460 nm with 200 ul of the standards and samples within 15 min.

Muscle Morphology

In order to evaluate muscle fiber cross-section area, LD muscle was sectioned at 10 μm thickness and stained with 10 μg/mL anti-dystrophin antibody (Sigma, D8168). Muscle fiber cross-sectional area was measured using ZenPro automated image analysis. Cross-sectional area of the LD was determined by tracing the loin at the 12^{th} rib and importing the image to ImageJ to calculate the area.

Satellite Cell isolation

Satellite cells were isolated (Alexander et al., 2012) and cultured (Chen et al., 2017) from individual pigs as previously described and were verified to be >95% Pax7+. Cryopreserved SCs from adequate treatment pigs were cultured in growth media containing DMEM + 10% Serum + antibiotics. The serum used in the media were serum collected from each of the pigs at days 11 and 17 and pooled according to treatment. Cells were grown at 37°C in a humidified environment containing 5% CO_2 in 15-cm plates coated with ECL Cell Attachment Matrix (MilliporeSigma, Burlington, MA) with complete media changes every other day until 80% confluence. Cells were then trypsinized and replated at a density of 3×10^3 cells/cm^2 for evaluation of proliferation, and at a density of 2.5×10^4 cells/cm^2 for determination of differentiation and miRNA analysis.

Satellite Cell Proliferation and Differentiation

For analysis of *in vitro* differentiation SCs were cultured in differentiation media (DMEM + 2% serum + antibiotics) for 3 d. At the end of differentiation cells were subjected to immunocytochemical staining for myosin heavy chain. Briefly, cells were fixed with 4% paraformaldehyde for 15 min and washed twice with ice-cold PBS. The samples were then incubated for 10 min with PBS containing 0.25% Triton X-100 (PBST) and washed in PBS 3 times for 5 min. Cells were blocked in 1% BSA in PBST, and incubated with mf20 antibody

(Developmental Studies Hybridoma Bank) overnight at 4° C. On the second day, samples were washed in PBS 3 times for 5 min, incubated with Dylight 488 second antibody (Thermo Fisher Scientific) for 1 h, and washed in PBS 3 times for 5 min. Nuclei were stained with 4′,6-diamidino-2- phenylindole (DAPI; Sigma-Aldrich) in PBS. Pictures were taken at 20× magnification. The total number of nuclei were counted, and the fusion index was presented as a ratio of nuclei within myotubes containing 2+ nuclei compared with the total nuclei. In vitro SC proliferation was determined at 0, 24, 48, and 72 h using the CyQUANT NF Cell Proliferation Assay Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions.

Gene Expression Analysis

Analysis of gene expression was performed as described previously (Rhoads et al., 2013). Briefly, total RNA was isolated from ~50 mg of *longissimus dorsi* tissue using TRIzol reagent (Invitrogen, Waltham, MA). Micro RNA was isolated from ~50 mg of *longissimus dorsi* tissue and satellite cells at day 0 and day 3 of differentiation using the mirVana™ miRNA Isolation Kit (Life Technologies, Carlsbad, CA) according to manufacturer's instructions. The eluted RNA and miRNA were quantified by absorbance at 260 nm and 1,000ng were reverse transcribed with iScript reverse transcriptase (Bio-Rad Laboratories, Hercules, CA). Micro RNA were reverse-transcribed using the stem loop method previously described by Kramer in 2011 by including a stem-loop primer in the reaction mix. Traditional PCR requires a template that is at least two times the length of the primers. Since miRNA's are 17-24 nucleotides in length, a stem loop primer is provided to bind to miRNA and increase their length to allow polymerase to bind and replicate. Real-time SYBR green PCR assays were performed with RNA and miRNA-specific primers (Table 3.1 and 3.2). PCR reactions were performed in a 20μl volume using SsoAdvanced universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). Reactions contained a 1-l primer

set and diluted cDNA (10 ng). Each sample was quantified in triplicate. SYBR Green fluorescence was quantified with the CFX96 TouchReal Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). A total of 40 PCR cycles were run, and a dissociation curve was included to verify the amplification of a single PCR product. Muscle tissue relative mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method with the average of RPL4 and 18S abundance used for normalization. A standard curve was generated with oligonucleotides of the mature miRNAs measured to quantify miRNA abundance. All primer sets were evaluated for adequate efficiency before use in this study.

Statistical analysis

Statistical analysis was performed in SAS (version 9.4; SAS Institute, Inc.). The normality for all data was tested using the Proc Univariate procedure of SAS. Data were transformed if necessary. Homogeneity of variance was evaluated by Levene's test. Body weight, average daily gain, feed efficiency, and were analyzed by Proc glimmix procedure with different diet treatments as the main effect. Tukey's test was used for multiple comparison tests if there is significant treatment effect. Proc mixed was used to analyze cross-sectional area. Proc mixed was used to analyze satellite cell proliferation and differentiation as repeated measures with day as the repeated effect. The compound symmetry, unstructured, toeplitz, variance components, and autoregressive 1 and heterogeneous versions of covariance structures were tested and the most appropriate (lowest Akaike's information criterion, Akaike's information criterion with correction, and Bayesian information criterion values) was used for each analysis. Model included treatment, day, and interactions.

Real time PCR data were analyzed using the $2^{-\Delta\Delta CT}$ method after normalization with the geometric mean of 18S and RPL4 expression. Gene expression was normalized back to the adequate treatment group for tissue gene expression and adequate treatment on day 0 for satellite

cell miRNA gene expression. The relative quantity (2-ΔΔCT) values were subjected to ANOVA analysis. Gene expression as analyzed by Proc mixed procedure with different diet treatments as the main effect. Tukey's test was used for pairwise comparison if there is significant diet or time effects.

Results

Growth Parameters

Protein is an efficient resource for the energy metabolism and muscle synthesis. Our study demonstrated that animal daily weight gain (D: 0.21±0.01; A: 0.24±0.01; E: 0.27±0.01), feed efficiency as indicated by average daily feed intake to average daily gain (D: 1.03±0.04; A: 0.89±0.05; E: 0.78±0.04), and final body weight (D: 4.75±0.27; A: 5.15±0.28; E: 5.60±0.27) were associated with crude protein level in the diet and all significantly higher in the Excess group than the Deficient group (Table 3.3). Feed efficiency in the adequate group was also significantly greater than the Deficient group. Changes in the phenotypic characters could be due to alternations in serum parameters responding to the differences in protein intake. Serum urea nitrogen is an indicator for global protein status. Our results indicated that dietary protein level significantly increased serum urea nitrogen level in a dose dependent manner at both d11 and d17 (Table 3.4). Protein is a great stimulator for IGF1 that promotes cell proliferation and differentiation, which is primarily secreted by the liver. Here, serum IGF1 increased with increasing protein level but didn't reach a significant level. Protein supplementation has been indicated to change serum phosphate and calcium level.

Muscle Morphology

Protein is a potent stimulator for muscle growth. Here we evaluated LD muscle cross-section area (CSA) and fiber CSA. There was significantly higher muscle fiber area in the Excess

than the Deficient group (E: 691.05±67.99 um^2; D: 462.07±28.36 um^2) (Figure 3.1). This is also observed in the total muscle cross sectional area of the LD in which Excess had a larger loin CSA than Deficient diets (E: 6.53±0.31 cm^2; D: 5.23±0.31) (P=0.02).

Satellite Cell Proliferation and Differentiation

Satellite cell proliferation was not different between treatments and time (Figure 3.2). To evaluate *in vitro* differentiation, fusion rates were evaluated by comparing the number of nuclei within a myotube containing 2+ nuclei with total nuclei present. An interaction between diet and time (P<0.01) occurred in which all treatments at day three had a lower myoblast fusion index than day 0 adequate and deficient diets but was not different from excess (Figure 3).

Gene Expression

There were no differences in expression between treatments for the myogenic genes MyoD, MyoG, myocyte enhancer factor 2 (MEF2A), and Pax7, as well as the negative regulator of muscle growth, MSTN (Figure 4). Excess treatments had a greater SDH expression level than Adequate and Excess treatments (P=0.076) (Figure 5). Excess treatments had a lower expression of IGF1 than adequate and deficient treatments (P=0.006). Deficient treatments had a lower expression of AMPK than adequate and excess treatments (P=0.04). Atrogin and PPARδ were not different between treatments (Figure 3.6). Deficient diets had the greatest expression of FOXO while excess diets had the lowest, and adequate diets intermediate and not different between the two (P=0.016). Deficient diets had greater expression of 4EBP1 than adequate and excess diets (P=0.005). Citrate synthase expression was greater in deficient pigs than excess pigs, with adequate being intermediate (P=0.035). Myosin heavy chain expression (MyHC) type-I was not different between treatments (Figure 3.7). Excess treatment had lower expression of MyHC-IIA than Adequate and Deficient treatments (P=0.003). MyHC-IIX tended to be different between treatments (P=0.08),

with adequate treatments having the higher expression than excess diets, with deficient diets being intermediate. Excess diets had higher expression of MyHC-IIB than adequate and deficient diets (P=0.002).

miRNA Expression

Longissimus dorsi tissue miRNA expression was not different between treatments for any miRNA analyzed (Figure 3.8). Satellite cell miRNA's -133a, -133b, - 206 did not differ between treatment. However, miR-1 had a tendency to have an interaction (P=0.06) between treatment and time, in which Excess treatment at day 0 had the highest expression while day 3 adequate had the lowest and all other treatment by time combinations were intermediate and not different from the two (Figure 3.9).

Discussion

Protein imbalance in early life has significant effect on animal health and growth. A previous study indicated that growth rate and feed efficiency were limited in neonatal pigs fed an ad libitum diet consisting of 10.2% of protein from 3 to 28 days of age, but improved as the protein level increased up to 22.4 percent (Becker et al., 1954). In sow milk, the protein level is about 27 % at dry matter basis, which could vary with the age and breeds of the sow (Pond et al., 1962). Since nutrition requirements in neonatal pigs have not been well documented, we have designed an experiment to evaluate impact of different protein levels on animal growth, feed efficiency, and bone development with protein requirement in the adequate group based on the sow milk composition. Our results suggested that animal growth performances were improved with the increase of protein supplementation; particularly, with 33% protein supplementation piglets had highest animal growth rate and feed efficiency. Increased protein intake increases amino acid oxidation and subsequent nitrogen excretion. Serum urea nitrogen concentration was significantly

increased from deficient to adequate and adequate to excess groups, which is positively correlated to our body weight gain. These results suggested that our excess group with 33% protein supplementation had greater protein utilization efficiency and optimal growth. On the other hand, what we considered adequate protein level (26%) is not sufficient for maximizing lean growth. Future practice could improve animal growth efficiency with higher protein level during the neonatal period.

Skeletal muscles are highly adaptive to environmental and nutritional changes via the adjustment of muscle volume and metabolic and fiber compositions. Dietary protein level is a great stimulator for muscle development. The same phenomenon has been found in our study, where the Excess treatments had significantly larger muscle fiber cross-section area than the deficient group, suggesting that high protein level increased muscle volume. This corresponded with increased LD muscle CSA. The relationship between nutrition and muscle fiber composition haven't been thoroughly studied, especially during neonatal development.

Pig skeletal muscle consists of four different fiber types (I, IIA, IIX, IIB) (Lefaucheur et al., 1998). These fiber types are classified by their myofibril ATPase, oxidative capacity, color, and size. Type I fibers are known as the most oxidative, have a small cross-sectional area, and are slow twitch, while type IIB fibers are large, glycolytic, and fast twitch. Types IIA and IIX are fast twitch oxidative-glycolytic intermediate to type I and IIB (Klont et al., 1998). Animals given a diet deficient in protein exhibited a greater expression of MyHC-IIA while excess protein had the greatest expression of MyHC-IIB. Type IIB fibers are generally larger than IIA. This corresponds with the differences observed in the fiber CSA with deficient animals having a smaller fiber CSA, and excess diets having the largest fiber CSA. This is consistent with previous reports of pigs fed high protein diets exhibiting a higher proportion of glycolytic fibers (Karlsson et al., 1993). Type

IIB fibers grow approximately twice as fast as type I fibers after birth, therefore, having a greater distribution of type IIB fibers positively correlated to body weight and muscle weight.(Oksbjerg et al., 1994; Choi and Kim, 2009).

Succinate dehydrogenase (SDH) is a marker of skeletal muscle oxidative capacity, which varies by fiber types depending on differences in energy demands and reliance on mitochondrial oxidative phosphorylation activities, such as type I > type 2A>type 2X>type 2B (Martin et al., 1985; Old and Johnson, 1989; Bloemberg and Quadrilatero, 2012). It helps to regulate metabolism through transcriptional control over metabolism-related genes in mitochondria and promotes metabolism of glucose and lipids. In the current study, the expression of SDH tended to be greater in excess pigs than deficient and adequate. Previous reports demonstrated that pigs fed a high protein diet may exhibit increased total respiration and ATP production (Adeola and Young, 1989).

Citrate synthase is a mitochondrial enzyme that is used as a marker for oxidative potential and is a key regulator of aerobic energy production in cells (Chaudhary et al., 1992). Citrate synthase activity is most closely related to type IIA fibers and exhibits a relationship ranking of type IIA > I > IIX > IIB (Delp and Duan, 1996). In the present study, citrate synthase expression was highest in deficient treatments and lowest in excess. This corresponds with the expression of MyHC-IIA being highest in deficient animals and lowest in excess. However, given that most enzymes are not regulated through transcription, drawing conclusions using SDH and citrate synthase as markers for fiber types warrants further investigation.

Slow oxidative fibers contain selectively active MEF2A proteins, and fibers transitioning from glycolytic fibers to oxidative had enhanced MEF2A activity; therefore, MEF2A may be responsible for the formation of slow-twitch fibers (Wu et al., 2000; Wu et al., 2001; Wu and Olson, 2002). Additionally, PPARd is higher in oxidative type I fibers compared with glycolytic

fibers (Wang et al., 2004). There were no differences in expression of MEF2A or PPARd which is consistent with a lack of differences observed in the expression of MyHC-I between treatments. Furthermore, myostatin has been suggested to regulate MEF2A expression. Absence of myostatin resulted in the downregulation of the MEF2A gene (Hennebry et al., 2009). In this study no differences in myostatin were observed between treatments.

The energy sensor, AMPK, maintains energy stores by refining anabolic and catabolic pathways, and its activation signifies a low energy balance in the cell (Hardie et al., 2012). In this study, deficient diets had decreased expression of AMPK compared to adequate and excess dietary treatments. The increase in AMPK in excess and adequate groups indicates greater energy demands in response to significant body weight gain. In addition, AMPK is involved in various singling pathways to regulate energy homeostasis such as lipogenesis and muscle protein synthesis. Translation initiation is regulated by eukaryotic initiation factor 4E binding proteins (4EBP1) through its binding and inhibition of eIF4E. Activity of 4EBP1's is regulated through its phosphorylation in response to changes in growth factors and nutrients (Haghighat et al., 1995; Khaleghpour et al., 1999). While its activity regulates translation initiation, total 4EBP1 can also influence translation control. Rats fed a sulfur amino acid-deficient diet had 2-4 fold increases in expression and protein abundance of 4EBP-1 (Sikalidis et al., 2013). Consistent with this, protein deficient diets in this study led to an upregulation of 4EBP-1 in muscle.

Muscle growth occurs when protein accretion exceeds protein degradation. When degradation surpasses accretion, atrophy occurs and is mediated by genes referred to as atrogenes. The expression of two atrogenes, MuRF1 and Atrogin-1, increase the most during muscle atrophy. Atrophy due to fasting increases Atrogin-1 8-40 fold (Bodine et al., 2001). In this study, dietary treatment did not lead to differences in MuRF-1 or Atrogin-1. However, FOXO promotes the

transcription of atrogenes. In the present study, FOXO1 was elevated in deficient diet pigs compared to excess pigs. This is consistent with previous findings that FOXO 1 and 3 mRNA levels rise in atrophying muscle due to fasting (Furuyama et al., 2003; Lecker et al., 2004). While the increase in FOXO-1 in deficient pigs was not enough to increase the expression of Atrogin-1, FOXO itself has been shown to be sufficient to cause atrophy of myotubes and mature muscle due to the increase in FOXO and may indicate atrophy and be contributing to the reduction in fiber CSA in pigs fed a protein deficient diet fibers (Sandri et al., 2004). Similarly, in humans, a short term protein restriction led to a reduction in transcription of genes associated with satellite cell proliferation and increased transcript levels of genes associated with the ubiquitin-dependent protein catabolism and apoptosis (Thalacker-Mercer et al., 2010).

It is widely accepted that IGF-I serum concentrations are positively correlated with growth rate (Lund-Larsen and Bakke, 1975; Whang et al., 2003; CHO et al., 2008). In agreement with previous studies, increasing dietary protein increased serum IGF-I; however, IGF-I expression was not increased in muscle tissue with increasing dietary protein. Furthermore, increased IGF-1 is correlated with increased growth rates also observed in this study.

Postnatal muscle growth through incorporation of satellite cells requires a highly coordinated process that requires precise expression of gene regulatory factors. Micro RNAs function to fine-tune the expression of genes by preventing translation and/or promote degradation of the target mRNA. Micro-RNAs play a key role in muscle development and satellite cell activity. Micro-RNA-133a enhances proliferation of satellite cells, while miR-1/206 promotes differentiation. During satellite cell differentiation, miR-1 and -206 are greatly upregulated (Chen et al., 2006) and target Pax7. Insulin-like Growth Factor-1 acts as both a target and a regulator of miR-1 through a feedback loop in which IGF-I binding to its receptor activates signaling pathways

that lead to the activation of AKT. Active AKT phosphorylates and inhibits the transcription factor Foxo3a, working to stimulate the expression of miR-1 (Elia et al., 2009). While miRNA expression in muscle tissue were not influenced by diet, they may still influence gene expression through their activity. In this study, miR-1 in satellite cells tended to have an interaction between diet and time. At day 0 of differentiation, excess dietary protein had the lowest myoblast fusion rate, however; at day 3 it was not different from adequate treatment. Interestingly, excess protein treatments had elevated miR-1 at day 0, which may contribute to the recovery of fusion rates at day 3 of differentiation.

Postnatal muscle growth relies on satellite cell incorporation to enhance the transcriptional capacity for future hypertrophy. Altering satellite cell function can lead to a reduction in growth potential. Previous *in vitro* studies report that branch chain amino acids and leucine availability can promote myocyte proliferation and differentiation (Averous et al., 2012; Dai et al., 2015; Duan et al., 2017). However, in this study varying dietary inclusion levels of protein did not influence satellite cell proliferation. A decrease in fusion is observed at day 3 of differentiation which may be due in part to myotubes beginning to contract and detach from the plate. While Deficient diets had the highest fusion rates at day 0, they also had the lowest expression of miR-1 at that timepoint. Consequently, at day 3, deficient diets had the lowest fusion rates, which may be due to the low expression of the differentiation miR-1 at day 0.

Conclusion

Taken together, the current study indicates that deficient protein supplementation is not enough for optimal animal growth. The Excess protein diet with 33% protein supplementation improved feed efficiency and body weight gain, which is largely due to muscle growth regulated by different genes at both transcriptional and translational levels. This study has practical

implications for future animal production and provides insights into further research on muscle growth and the roles of satellite cells in muscle development.

Acknowledgments

This study was funded in part by USDA (2015-67015-25094).

References

Adeola, O., and L. G. Young. 1989. Dietary Protein-Induced Changes in Porcine Muscle Respiration, Protein Synthesis and Adipose Tissue Metabolism. Journal of Animal Science 67(3):664-673. doi: 10.2527/jas1989.673664x

Alexander, L. S., B. S. Seabolt, R. P. Rhoads, and C. H. Stahl. 2012. Neonatal phosphate nutrition alters in vivo and in vitro satellite cell activity in pigs. Nutrients 4(6):436-448. doi: 10.3390/nu4060436

Allen, R. E., R. A. Merkel, and R. B. Young. 1979. Cellular aspect of muscle growth: myogenic cell proliferation. Journal of animal science 49(1):115-127.

Averous, J., J. C. Gabillard, I. Seiliez, and D. Dardevet. 2012. Leucine limitation regulates myf5 and myoD expression and inhibits myoblast differentiation. Experimental Cell Research 318(3):217-227. doi: 10.1016/j.yexcr.2011.10.015

Becker, D. E., D. E. Ullrey, and S. W. Terrill. 1954. Protein and Amino Acid Intakes for Optimum Growth Rate in the Young Pig. Journal of Animal Science 13(2):346-356.

Bloemberg, D., and J. Quadrilatero. 2012. Rapid determination of myosin heavy chain expression in rat, mouse, and human skeletal muscle using multicolor immunofluorescence analysis. PLoS One 7(4):e35273. doi: 10.1371/journal.pone.0035273

Bodine, S. C., E. Latres, S. Baumhueter, V. K.-M. Lai, L. Nunez, B. A. Clarke, W. T. Poueymirou, F. J. Panaro, E. Na, K. Dharmarajan, Z.-Q. Pan, D. M. Valenzuela, T. M. DeChiara, T. N. Stitt, G. D. Yancopoulos, and D. J. Glass. 2001. Identification of Ubiquitin Ligases Required for Skeletal Muscle Atrophy. Science 294(5547):1704-1708. doi: 10.1126/science.1065874

Chaudhary, R., B. P. Chowdhary, I. Harbitz, I. Gustavsson, and C. T. Evans. 1992. Localization of the citrate synthase (CS) gene to the p12-p13 bands of chromosome 5 in pigs by in situ hybridization. Hereditas 117(1):39-43. doi: 10.1111/j.1601-5223.1992.tb00005.x

Chen, J.-F., E. M. Mandel, J. M. Thomson, Q. Wu, T. E. Callis, S. M. Hammond, F. L. Conlon, and D.-Z. Wang. 2006. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nature genetics 38(2):228.

Chen, Y., H. Zhu, S. R. McCauley, L. Zhao, S. E. Johnson, R. P. Rhoads, and S. W. El-Kadi. 2017. Diminished satellite cell fusion and S6K1 expression in myotubes derived from skeletal muscle of low birth weight neonatal pigs. Physiological Reports 5(3):e13075. doi: 10.14814/phy2.13075

CHO, J. H., Y. J. CHEN, B. J. MIN, J. S. YOO, Y. WANG, and I. H. KIM. 2008. Effects of reducing dietary crude protein on growth performance, odor gas emission from manure and blood urea nitrogen and IGF-1 concentrations of serum in nursery pigs. Animal Science Journal 79(4):453-459. doi: 10.1111/j.1740-0929.2008.00549.x

Choi, Y. M., and B. C. Kim. 2009. Muscle fiber characteristics, myofibrillar protein isoforms, and meat quality. Livestock Science 122(2):105-118. doi: 10.1016/j.livsci.2008.08.015

Dai, J.-M., M.-X. Yu, Z.-Y. Shen, C.-Y. Guo, S.-Q. Zhuang, and X.-S. Qiu. 2015. Leucine promotes proliferation and differentiation of primary preterm rat satellite cells in part through mTORC1 signaling pathway. Nutrients 7(5):3387-3400. doi: 10.3390/nu7053387

Davis, T. A., D. G. Burrin, M. L. Fiorotto, and H. V. Nguyen. 1996. Protein synthesis in skeletal muscle and jejunum is more responsive to feeding in 7-than in 26-day-old pigs. American

Journal of Physiology-Endocrinology and Metabolism 270(5):E802-E809. doi: 10.1152/ajpendo.1996.270.5.E802
Davis, T. A., and M. L. Fiorotto. 2009. Regulation of muscle growth in neonates. Curr Opin Clin Nutr Metab Care 12(1):78-85. doi: 10.1097/MCO.0b013e32831cef9f
Davis, T. A., M. L. Fiorotto, D. G. Burrin, W. G. Pond, and H. V. Nguyen. 1997. Intrauterine growth restriction does not alter response of protein synthesis to feeding in newborn pigs. American Journal of Physiology-Endocrinology and Metabolism 272(5):E877-E884. doi: 10.1152/ajpendo.1997.272.5.E877
Davis, T. A., M. L. Fiorotto, H. V. Nguyen, and P. J. Reeds. 1989. Protein turnover in skeletal muscle of suckling rats. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 257(5):R1141-R1146. doi: 10.1152/ajpregu.1989.257.5.R1141
Delp, M. D., and C. Duan. 1996. Composition and size of type I, IIA, IID/X, and IIB fibers and citrate synthase activity of rat muscle. Journal of Applied Physiology 80(1):261-270. doi: 10.1152/jappl.1996.80.1.261
Duan, Y., L. Zeng, F. Li, W. Wang, Y. Li, Q. Guo, Y. Ji, B. e. Tan, and Y. Yin. 2017. Effect of branched-chain amino acid ratio on the proliferation, differentiation, and expression levels of key regulators involved in protein metabolism of myocytes. Nutrition 36:8-16. doi: 10.1016/j.nut.2016.10.016
Elia, L., R. Contu, M. Quintavalle, F. Varrone, C. Chimenti, M. A. Russo, V. Cimino, L. De Marinis, A. Frustaci, and D. Catalucci. 2009. Reciprocal regulation of microRNA-1 and IGF-1 signal transduction cascade in cardiac and skeletal muscle in physiological and pathological conditions. Circulation 120(23):2377. doi: 10.1161/CIRCULATIONAHA.109.879429
Furuyama, T., K. Kitayama, H. Yamashita, and N. Mori. 2003. Forkhead transcription factor FOXO1 (FKHR)-dependent induction of PDK4 gene expression in skeletal muscle during energy deprivation. Biochemical Journal 375(2):365-371. doi: 10.1042/bj20030022
Haghighat, A., S. Mader, A. Pause, and N. Sonenberg. 1995. Repression of cap-dependent translation by 4E-binding protein 1: competition with p220 for binding to eukaryotic initiation factor-4E. The EMBO Journal 14(22):5701-5709. doi: 10.1002/j.1460-2075.1995.tb00257.x
Hamilton, A. J., and D. C. Baulcombe. 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286(5441):950-952.
Hardie, D. G., F. A. Ross, and S. A. Hawley. 2012. AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nature Reviews Molecular Cell Biology 13(4):251-262. doi: 10.1038/nrm3311
Hennebry, A., C. Berry, V. Siriett, P. O'Callaghan, L. Chau, T. Watson, M. Sharma, and R. Kambadur. 2009. Myostatin regulates fiber-type composition of skeletal muscle by regulating MEF2 and MyoD gene expression. American Journal of Physiology-Cell Physiology 296(3):C525-C534. doi: 10.1152/ajpcell.00259.2007
Karlsson, A., A.-C. Enfält, B. Essén-Gustavsson, K. Lundström, L. Rydhmer, and S. Stern. 1993. Muscle histochemical and biochemical properties in relation to meat quality during selection for increased lean tissue growth rate in pigs. Journal of Animal Science 71(4):930-938. doi: 10.2527/1993.714930x
Khaleghpour, K., S. Pyronnet, A.-C. Gingras, and N. Sonenberg. 1999. Translational Homeostasis: Eukaryotic Translation Initiation Factor 4E Control of 4E-Binding Protein

1 and p70 S6 Kinase Activities. Molecular and Cellular Biology 19(6):4302-4310. doi: 10.1128/mcb.19.6.4302
Klont, R. E., L. Brocks, and G. Eikelenboom. 1998. Muscle fibre type and meat quality. Meat Science 49:S219-S229. doi: 10.1016/S0309-1740(98)90050-X
Kramer, M. F. 2011. Stem-loop RT-qPCR for miRNAs. Current protocols in molecular biology 95(1):15.10. 11-15.10. 15. doi: 10.1002/0471142727.mb1510s95
Krol, J., K. Sobczak, U. Wilczynska, M. Drath, A. Jasinska, D. Kaczynska, and W. J. Krzyzosiak. 2004. Structural features of microRNA (miRNA) precursors and their relevance to miRNA biogenesis and small interfering RNA/short hairpin RNA design. Journal of Biological Chemistry 279(40):42230-42239.
Laurent, G., R. McAnulty, and J. Gibson. 1985. Changes in collagen synthesis and degradation during skeletal muscle growth. American Journal of Physiology-Cell Physiology 249(3):C352-C355. doi: 10.1152/ajpcell.1985.249.3.C352
Lecker, S. H., R. T. Jagoe, A. Gilbert, M. Gomes, V. Baracos, J. Bailey, S. R. Price, W. E. Mitch, and A. L. Goldberg. 2004. Multiple types of skeletal muscle atrophy involve a common program of changes in gene expression. The FASEB Journal 18(1):39-51. doi: 10.1096/fj.03-0610com
Lefaucheur, L., R. Hoffman, D. Gerrard, C. Okamura, N. Rubinstein, and A. Kelly. 1998. Evidence for three adult fast myosin heavy chain isoforms in type II skeletal muscle fibers in pigs. Journal of animal science 76(6):1584-1593. doi: 10.2527/1998.7661584x
Li, Y., F. Li, L. Wu, H. Wei, Y. Liu, T. Li, B. Tan, X. Kong, K. Yao, S. Chen, F. Wu, Y. Duan, and Y. Yin. 2016. Effects of dietary protein restriction on muscle fiber characteristics and mTORC1 pathway in the skeletal muscle of growing-finishing pigs. J Anim Sci Biotechnol 7(1):47. doi: 10.1186/s40104-016-0106-8
Lund-Larsen, T. R., and H. Bakke. 1975. Growth Hormone and Somatomedin Activities in Lines of Pigs Selected for Rate of Gain and Thickness of Backfat. Acta Agriculturae Scandinavica 25(3):231-234. doi: 10.1080/00015127509435044
Martin, T. P., A. C. Vailas, J. B. Durivage, V. R. Edgerton, and K. R. Castleman. 1985. Quantitative histochemical determination of muscle enzymes: biochemical verification. J Histochem Cytochem 33(10):1053-1059. doi: 10.1177/33.10.4045183
McCracken, K. J., S. M. Eddie, and W. G. Stevenson. 1980. Energy and protein nutrition of early-weaned pigs. 1. effect of energy intake and energy: protein on growth, efficiency and nitrogen utilization of pigs between 8–32 d. British Journal of Nutrition 43(2):289-304. doi: 10.1079/BJN19800092
Miersch, C., K. Stange, S. Hering, M. Kolisek, T. Viergutz, and M. Röntgen. 2017. Molecular and functional heterogeneity of early postnatal porcine satellite cell populations is associated with bioenergetic profile. Scientific Reports 7(1):1-14.
Mozdziak, P., E. Schultz, and R. Cassens. 1997. Myonuclear accretion is a major determinant of avian skeletal muscle growth. American Journal of Physiology-Cell Physiology 272(2):C565-C571. doi: 10.1152/ajpcell.1997.272.2.C565
NRC, N. R. C. 2012. Nutrient requirements of swine. National Academies Press.
Oksbjerg, N., P. Henckel, and T. Rolph. 1994. Effects of Salbutamol, a β2-Adrenergic Agonist, on Muscles of Growing Pigs Fed Different Levels of Dietary Protein: I. Muscle Fibre Properties and Muscle Protein Accretion. Acta Agriculturae Scandinavica, Section A — Animal Science 44(1):12-19. doi: 10.1080/09064709409410176

Old, S. L., and M. A. Johnson. 1989. Methods of microphotometric assay of succinate dehydrogenase and cytochrome c oxidase activities for use on human skeletal muscle. Histochem J 21(9-10):545-555.

Pond, W. G., L. D. Vanvleck, and D. A. Hartman. 1962. Parameters for Milk Yield and for Percents of Ash, Dry Matter, Fat and Protein in Sows. Journal of Animal Science 21(2):293-&.

Reinhart, B. J., F. J. Slack, M. Basson, A. E. Pasquinelli, J. C. Bettinger, A. E. Rougvie, H. R. Horvitz, and G. Ruvkun. 2000. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. nature 403(6772):901.

Rhoads, R., K. Flann, T. Cardinal, C. Rathbone, X. Liu, and R. E. Allen. 2013. Satellite cells isolated from aged or dystrophic muscle exhibit a reduced capacity to promote angiogenesis in vitro. Biochemical and biophysical research communications 440(3):399-404. doi: 10.1016/j.bbrc.2013.09.085

Sandri, M., C. Sandri, A. Gilbert, C. Skurk, E. Calabria, A. Picard, K. Walsh, S. Schiaffino, S. H. Lecker, and A. L. Goldberg. 2004. Foxo Transcription Factors Induce the Atrophy-Related Ubiquitin Ligase Atrogin-1 and Cause Skeletal Muscle Atrophy. Cell 117(3):399-412. doi: 10.1016/S0092-8674(04)00400-3

Sikalidis, A. K., K. M. Mazor, M. Kang, H. Liu, and M. H. Stipanuk. 2013. Total 4EBP1 Is Elevated in Liver of Rats in Response to Low Sulfur Amino Acid Intake. Journal of Amino Acids 2013:864757. doi: 10.1155/2013/864757

Thalacker-Mercer, A. E., J. C. Fleet, B. A. Craig, and W. W. Campbell. 2010. The skeletal muscle transcript profile reflects accommodative responses to inadequate protein intake in younger and older males. The Journal of Nutritional Biochemistry 21(11):1076-1082. doi: 10.1016/j.jnutbio.2009.09.004

Wang, Y.-X., C.-L. Zhang, R. T. Yu, H. K. Cho, M. C. Nelson, C. R. Bayuga-Ocampo, J. Ham, H. Kang, and R. M. Evans. 2004. Regulation of Muscle Fiber Type and Running Endurance by PPARδ. PLOS Biology 2(10):e294. doi: 10.1371/journal.pbio.0020294

Whang, K. Y., S. W. Kim, S. M. Donovan, F. K. McKeith, and R. A. Easter. 2003. Effects of protein deprivation on subsequent growth performance, gain of body components, and protein requirements in growing pigs. J Anim Sci 81(3):705-716. doi: 10.2527/2003.813705x

Wu, H., F. J. Naya, T. A. McKinsey, B. Mercer, J. M. Shelton, E. R. Chin, A. R. Simard, R. N. Michel, R. Bassel-Duby, E. N. Olson, and R. S. Williams. 2000. MEF2 responds to multiple calcium-regulated signals in the control of skeletal muscle fiber type. The EMBO Journal 19(9):1963-1973. doi: 10.1093/emboj/19.9.1963

Wu, H., and E. N. Olson. 2002. Activation of the MEF2 transcription factor in skeletal muscles from myotonic mice. The Journal of Clinical Investigation 109(10):1327-1333. doi: 10.1172/JCI15417

Wu, H., B. Rothermel, S. Kanatous, P. Rosenberg, F. J. Naya, J. M. Shelton, K. A. Hutcheson, J. M. DiMaio, E. N. Olson, R. Bassel-Duby, and R. S. Williams. 2001. Activation of MEF2 by muscle activity is mediated through a calcineurin-dependent pathway. The EMBO Journal 20(22):6414-6423. doi: 10.1093/emboj/20.22.6414

Tables and Figures

Table 3.1 Nucleotide sequences for real-time PCR primers

Target	Direction	Sequence
18S	Forward	GTAACCCGTTGAACCCCAT
	Reverse	CCA TCC AAT CGG TAG TAG CG
AMPKinase	Forward	ACGGAGCAAGTAACCCCAGC
	Reverse	GCCACGAGGATGACGATGAA
Atrogin-1	Forward	CAGCTCACATCCCTGAGTGG
	Reverse	GACTTGCCGACTCTCTGGAC
FOXO1	Forward	CAAGAGCGTGCCCTACTTCA
	Reverse	GGTTGAGCATCCACCAGGAA
IGF-1	Forward	GCACATCACATCCTCTTCGC
	Reverse	GCCTCCTCAGATCACAGCTC
MEF2AA	Forward	TGAATACCCAGAGGATAAGCAGTT
	Reverse	TAATCGGTGTTGTAGGCGG
MyHC I	Forward	AAGGGCTTGAACGAGGAGTAGA
	Reverse	TTATTCTGCTTCCTCCAAAGGG
MyHC IIa	Forward	GCTGAGCGAGCTGAAATCC
	Reverse	ACTGAGACACCAGAGCTTCT
MyHC IIb	Forward	ATGAAGAGGAACCACATTA
	Reverse	TTATTGCCTCAGTAGCTTG
MyHC IIx	Forward	AGAAGATCAACTGAGTGAACT
	Reverse	AGAGCTGAGAAACTAACGTG
MSTN	Forward	CCAGAGAGATGACAGCAGTGATG
	Reverse	TTCCTTCCACTTGCATTAGAAGATC
Murf-1	Forward	CATGTGCAAGGAGCACGAAG
	Reverse	TGGAGATGCGGTTACTCAGC
MyoD	Forward	GCGTGCAAACGCAAGACCACTAA
	Reverse	AGTCTCGAAGGCCTCGTTGACTTT
MyoG	Forward	TGACCCTACAGATGCCCACAATCT
	Reverse	GTTGGGCATGGTTTCATCTGGGAA
Pax7	Forward	CAACCACATCCGCCACAAGATAGT
	Reverse	AGAGGATCTTGGAGACACAGCCAT
PPARd	Forward	GAAGAACCGCAACAAGTGCC
	Reverse	TTCCTTTTCTCTGCCTCGGG
RPL4	Forward	AGGAGGCTGTTCTGCTTCTG
	Reverse	CCTTCAGAAACATCCCTGGA
SDH	Forward	TGGCCGGCACAGCTCCCAAT
	Reverse	GCACGTTCCCTGCTCTCCCG

Table 3.2 Nucleotide sequences for miRNA real-time PCR primers

Target	Primer	Primer sequences (5'-3')
ssc-miR-1	Stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTACATA
	Forward	GGCGGCTGGAATGTAAAGAAG
	Reverse	CAGTGCAGGGTCCGAGGTAT
	Oligonucleotide	TGGAATGTAAAGAAGTATGTA
ssc-miR-133a-3p	Stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGCTG
	Forward	TTGCGTTGGTCCCCTTCA
	Reverse	CAGTGCAGGGTCCGAGGTAT
	Oligonucleotide	TTGGTCCCCTTCAACCAGCTG
ssc-miR-133b	Stem-loop	GCGTGGTCCACACCACCTGAGCCGCCACGACCACGCATAGCTGG
	Forward	CCAGCCTTTGGTCCCCTTC
	Reverse	TCCACACCACCTGAGCCG
	Oligonucleotide	TTTGGTCCCCTTCAACCAGCTAT
ssc-miR-206	Stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCACAC
	Forward	CCGCGTGGAATGTAAGGAAGT
	Reverse	CAGTGCAGGGTCCGAGGTAT
	Oligonucleotide	TGGAATGTAAGGAAGTGTGTGA

Table 3.3 Impact of deficient, adequate, and excess protein levels on growth performance of neonatal pigs[1]

	Deficient	Adequate	Excess
Initial BW (kg)	1.47±0.10	1.46±0.10	1.46±0.10
Final BW (kg)	4.75±0.27[b]	5.15±0.28[ab]	5.60±0.27[a]
ADG (kg)[1]	0.21±0.01[b]	0.24±0.01[ab]	0.27±0.01[a]
FCR[2]	1.03±0.04[a]	0.89±0.05[b]	0.78±0.04[b]

[1] Values are means ± SEMs. Labeled means in a row without a common letter differ, $P < 0.05$ (a > b)

[2]Average Daily Gain

[3]Feed Conversion Ratio calculated by weight gain (kg)/ feed consumed (liquid, g)

Table 3.4 Effects of dietary protein levels on serum parameters in neonatal pigs[1]

		Deficient	Adequate	Excess
Urea Nitrogen	Day11	2.81±0.40[c]	6.50±0.42[b]	12.36±0.40[a]
	Day17	3.87±0.40[c]	6.77±0.40[b]	12.06±0.38[a]
IGF1 level (ng/ml)	Day11	96.3±10.0	115.6±10.0	120.2±10.0
	Day17	153.1±14.3	172.9±14.3	195.6±14.3
Phosphate (mg/dL)	Day11	10.37±0.24	10.45±0.22	10.38±0.27
	Day17	10.98±0.23	11.46±0.24	11.99±0.22

[1] Values are means ± SEMs. Labeled means in a row without a common letter differ, $P < 0.05$ (a > b). IGF1, Insulin-like Growth Factor-1.

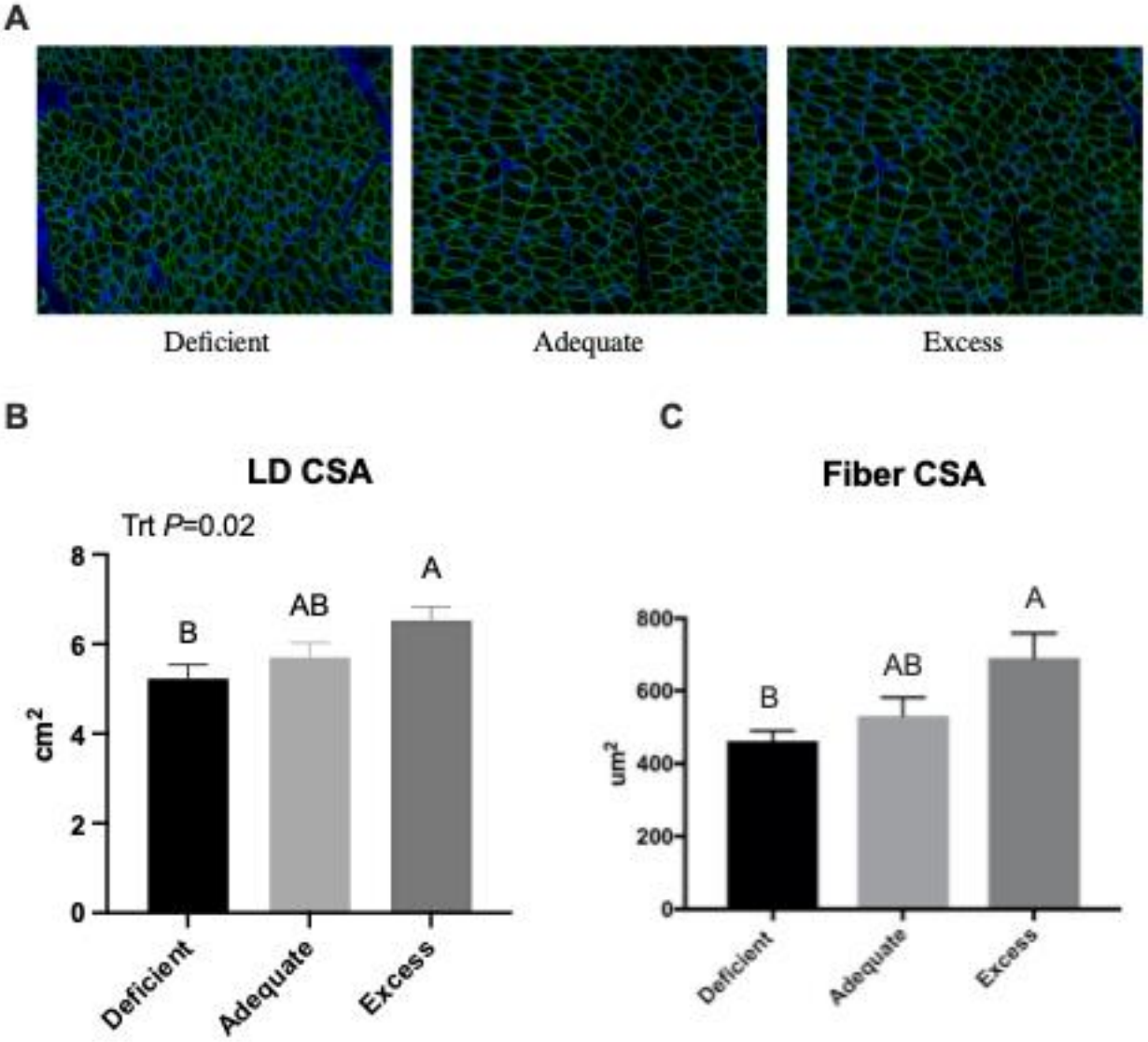

Figure 3.1 Cross sectional area analysis of the *longissimus dorsi* muscle and fibers of pigs fed differing levels of dietary protein. Muscle cross sectional area (B), fiber cross sectional area (C) and representative images of fiber cross sectional area (A) of pigs fed a diet containing either 25% deficient protein, adequate protein, or a 25% excess diet. Results are means ± SEM. Means without a common letter are different at $P<0.05$.

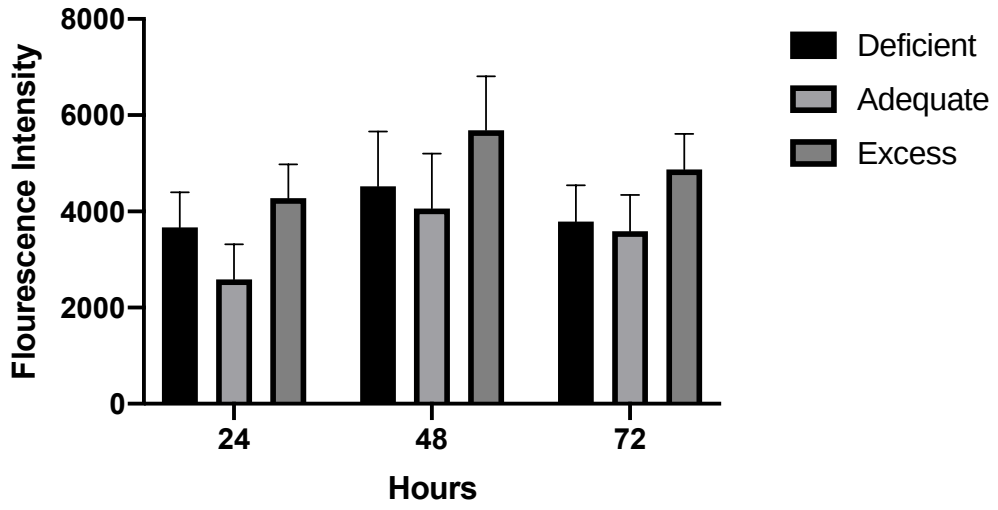

Figure 3.2 Satellite cell proliferation of pigs fed a diet containing either 25% deficient protein, adequate protein, or a 25% excess diet at 24, 48, and 72 hours. Results are means ± SEM.

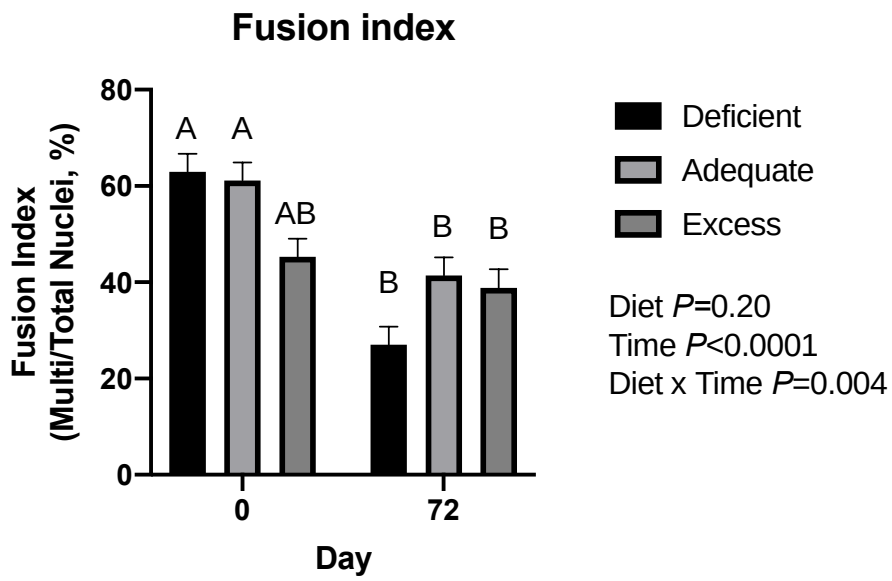

Figure 3.3 Satellite cell differentiation from the *longissimus dorsi* muscle of pigs fed varying levels of dietary protein. Fusion index expressed as the percentage of total nuclei that are located in myotubes of satellite cells of pigs fed a diet containing either 25% deficient protein, adequate protein, or a 25% excess protein diet. Values are means ± SEMs. Means at a time without a common letter differ $P < 0.05$.

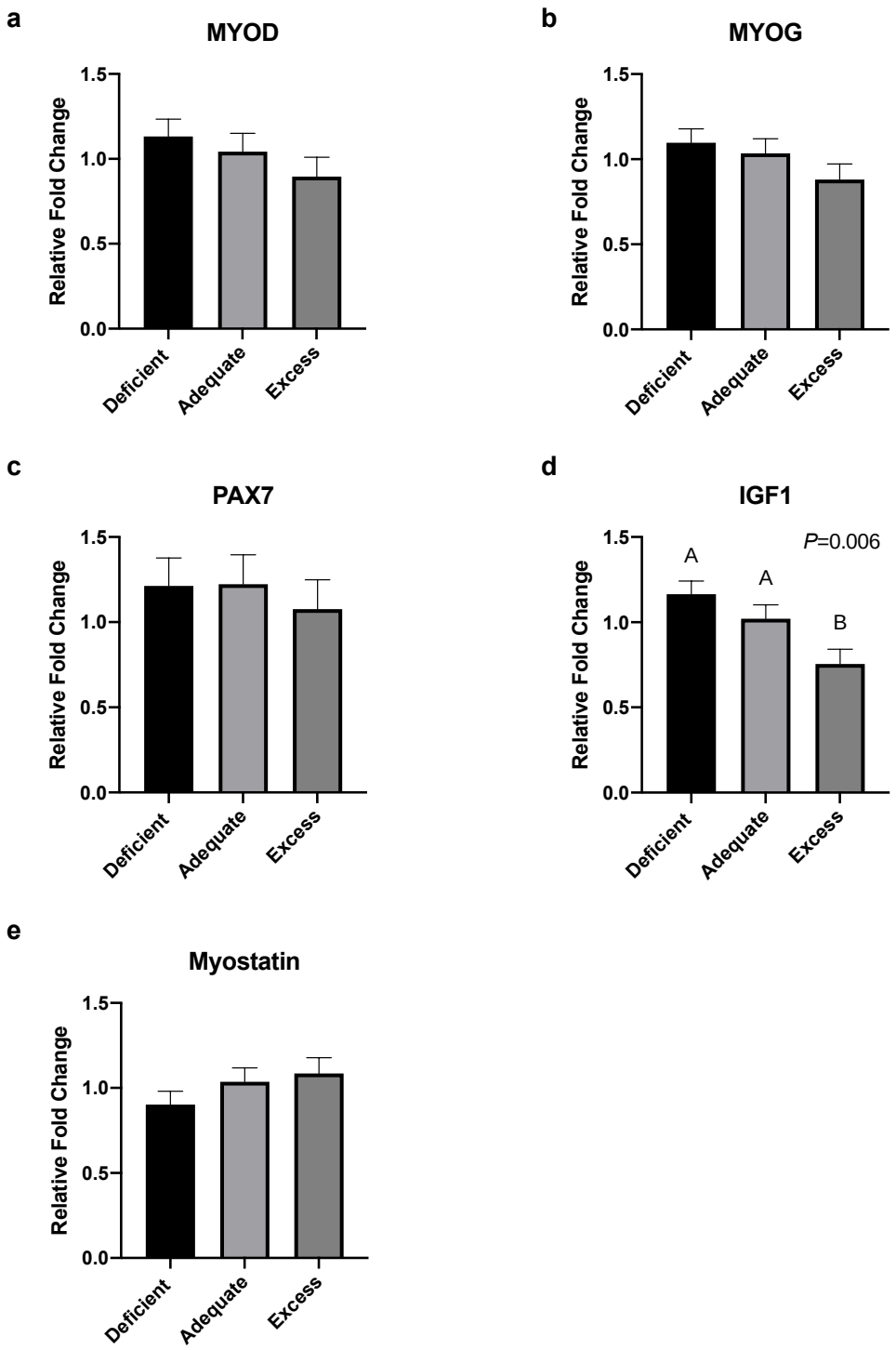

Figure 3.4 mRNA expression of genes involved in muscle growth in the *longissimus dorsi* muscle of pigs fed differing levels of protein. Pigs were fed a diet containing either 25% deficient protein, adequate protein, or a 25% excess diet. Results are means ± SEM. Means without a common letter are different at $P<0.05$.

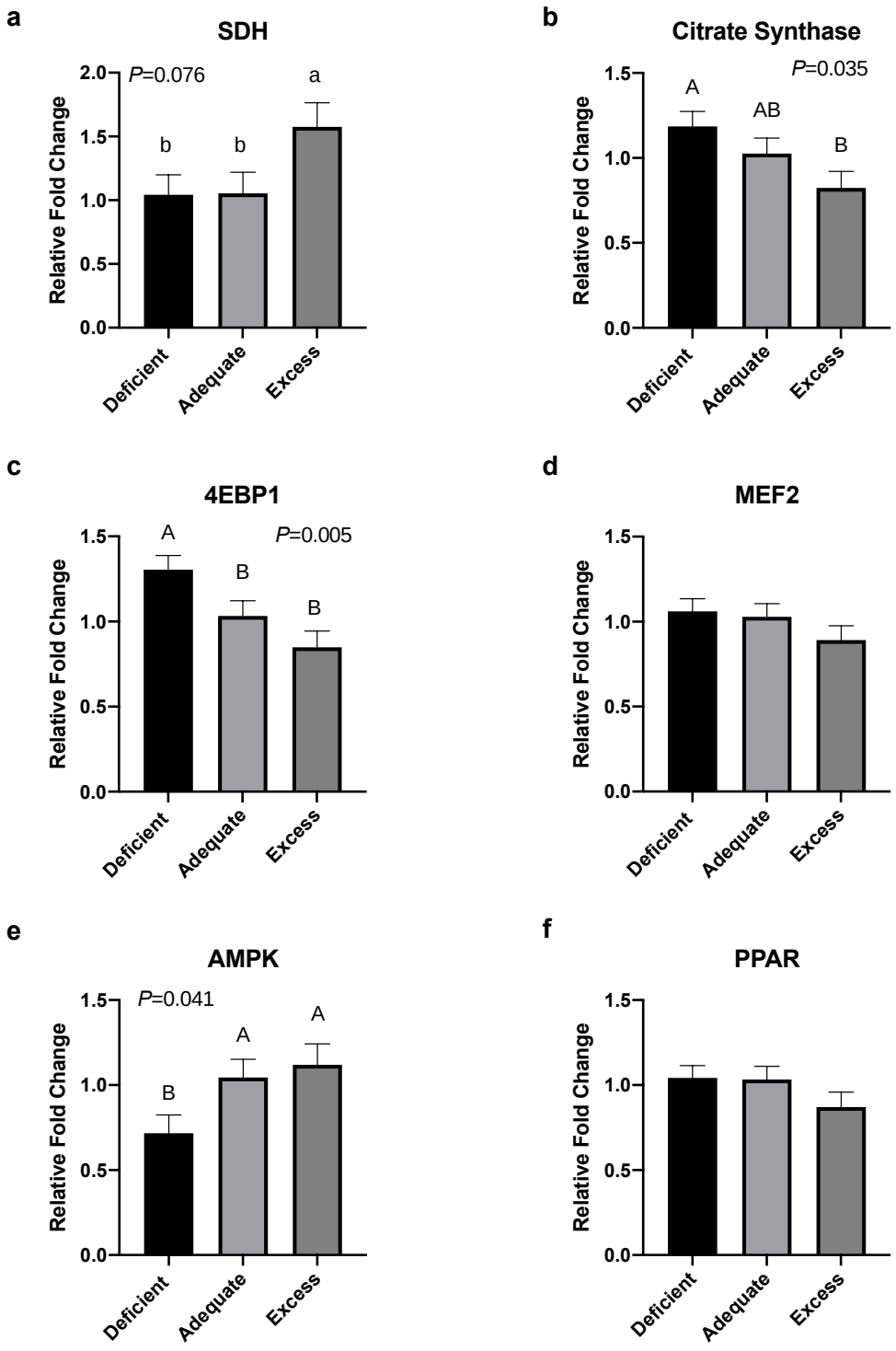

Figure 3.5 mRNA expression of genes involved in energy sensing in the *longissimus dorsi* muscle of pigs fed differing levels of protein. Pigs were fed a diet containing either 25% deficient protein, adequate protein, or a 25% excess diet. Results are means ± SEM. Means without a common uppercase letter are different at $P<0.05$ (A>B). Means without a common lowercase letter are different at $P<0.10$ (a>b).

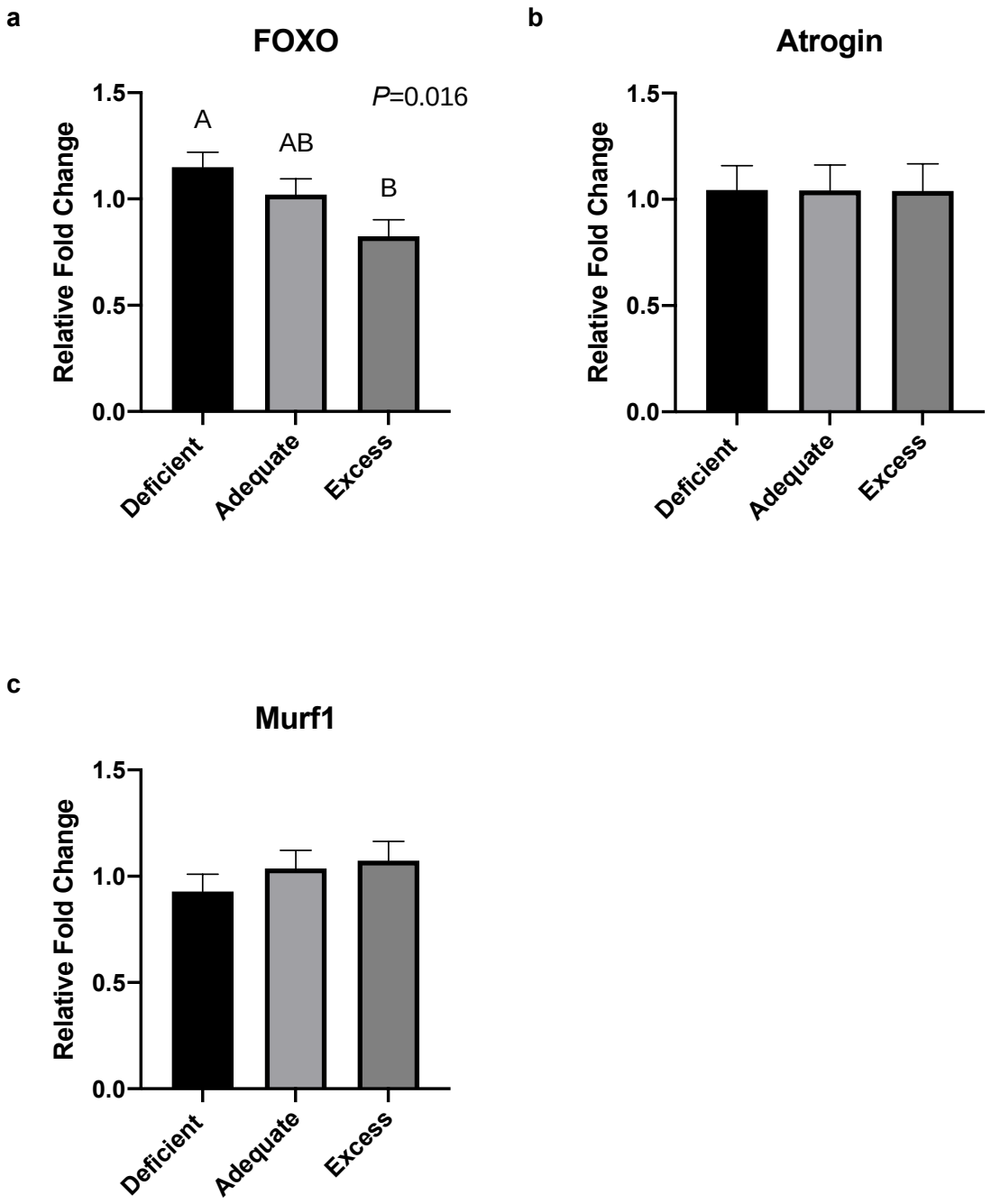

Figure 3.6 mRNA expression of genes involved in muscle degradation in the *longissimus dorsi* muscle of pigs fed differing levels of protein. Pigs were fed a diet containing either 25% deficient protein, adequate protein, or a 25% excess diet. Results are means ± SEM. Means without a common letter are different at P<0.05.

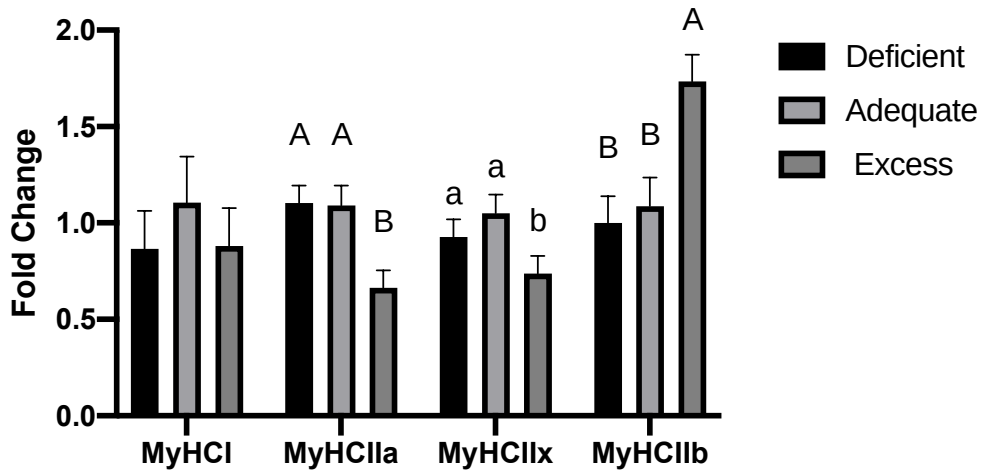

Figure 3.7 mRNA expression of the myosin heavy chain (MyHC) isoforms in the *longissimus dorsi* muscle of pigs fed differing levels of protein. Pigs were fed a diet containing either 25% deficient protein, adequate protein, or a 25% excess diet. Results are means ± SEM. Means without a common uppercase letter are different at $P<0.05$. Means without a common lowercase letter are different at $P<0.10$.

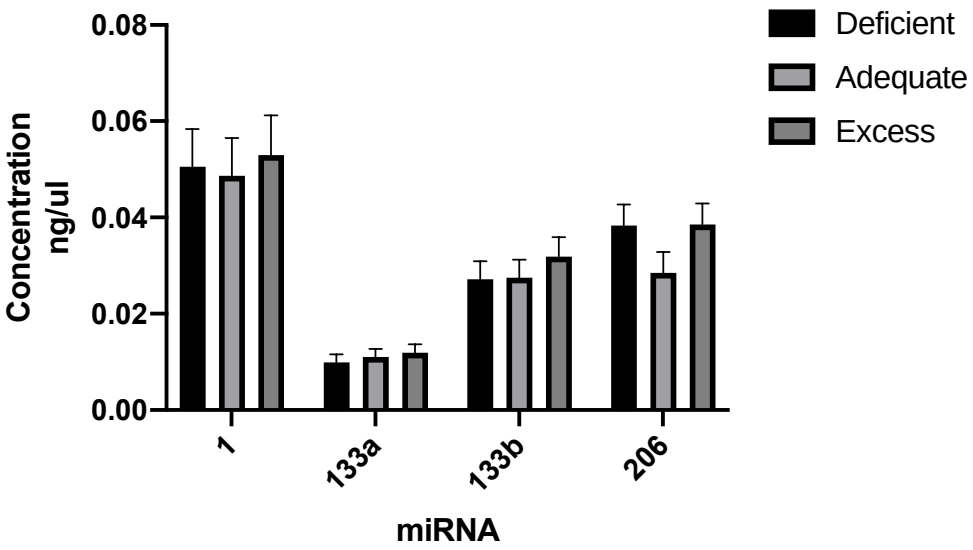

Figure 3.8 Expression of miRNA in the *longissimus dorsi* muscle of pigs fed differing levels of protein. Pigs were fed a diet containing either 25% deficient protein, adequate protein, or a 25% excess diet. Results are means ± SEM. Means without a common letter are different at $P<0.05$.

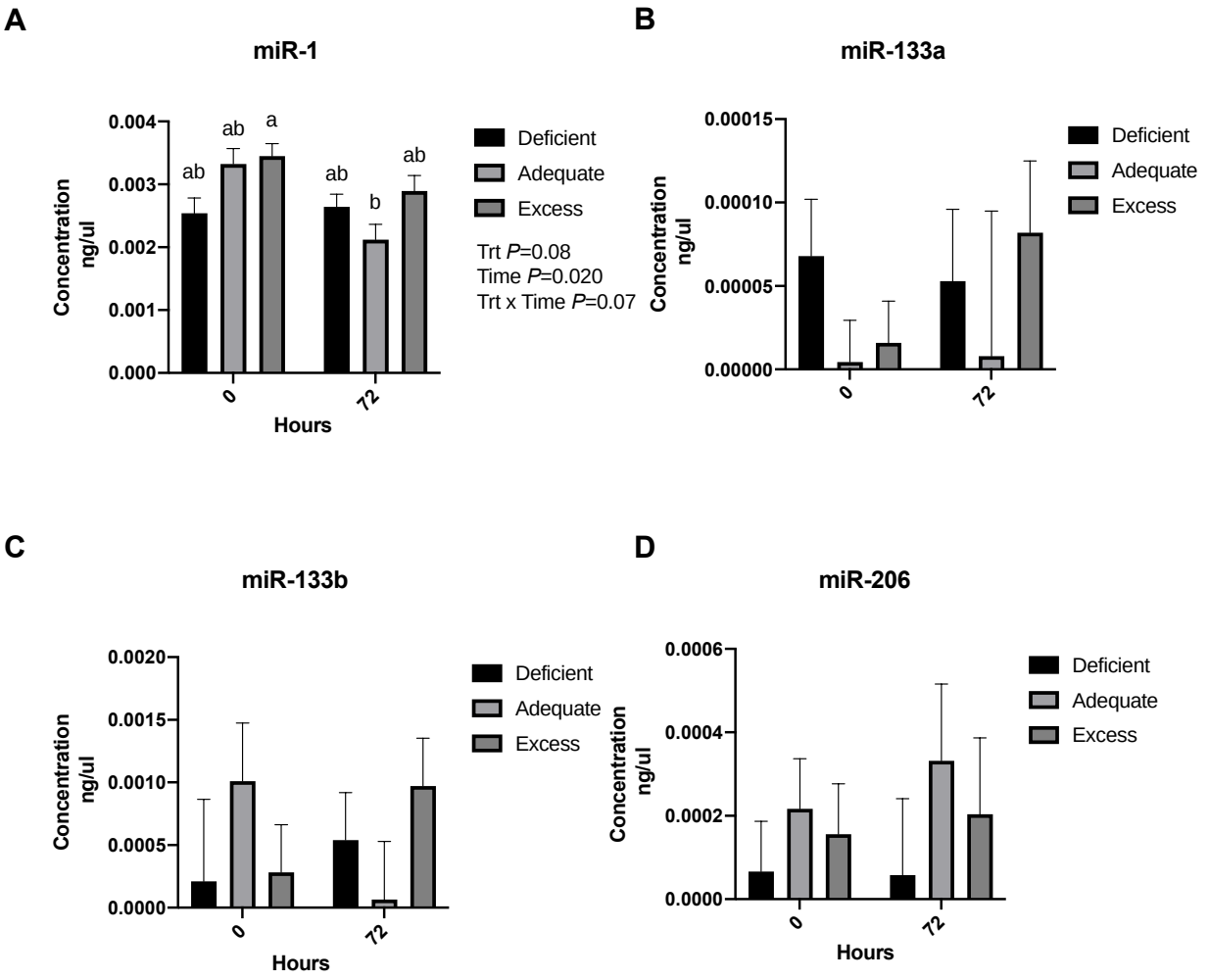

Figure 3.9 Expression of miR-1 (A), miR-133a (B), miR-133b (C), and miR-206 (D) in satellite cells of pigs fed differing levels of protein. Pigs were fed a diet containing either 25% deficient protein, adequate protein, or a 25% excess diet at 0 hours and 72 hours of differentiation. Results are means ± SEM. Means without a common lowercase letter are different at $P<0.10$.

Chapter 4: Caloric Intake Affects Energy Metabolism and Satellite Cell Fusion Through Micro-RNA Expression in Neonatal Pigs

Abstract

Growth as a percentage of body weight is highest during the neonatal period and restricting neonatal nutrition can lead to permanent muscle growth deficits. Nutrient restriction during the neonatal period has been shown to decrease growth rate by altering satellite cell function. Insufficient muscle development during early postnatal period can permanently alter later growth, muscle contraction and metabolic maturation. Furthermore, undernutrition during this period affects muscle growth more so than overall body weight, suggesting that more vital tissues have higher priority during restriction. Energy metabolism is altered by postnatal undernutrition through reduced glycolytic capacity and increased potential for β-oxidation. Conversely, high nutritional intake has the ability to induce oxidative stress. This study sought to discern the impact of dietary energy balance on muscle growth during the neonatal period. Twenty-six piglets obtained at 1 day of age received either an adequate energy diet, a 25% deficient diet, or a 25% excess energy diet over a 17-day trial. Piglet starting weights were 1.36kg ±0.09 for deficient and 1.34kg±0.09 for adequate and 1.33kg±0.09 for excess treatments. Final body weight and ADG were not different between treatments. However, feed efficiency improved with energy balance with Excess diets being the most efficient, and deficient diets being the least (P<0.001). Histology analysis indicated that LD muscle and fiber cross-sectional area were not different between treatments. Postnatal skeletal muscle growth requires satellite cell activation and fusion with existing myofibers. Delay or absence of satellite cell proliferation and differentiation leads to a reduction in postnatal muscle hypertrophy and reduced growth potential. Satellite cell proliferation was not different between treatments. As expected, proliferation increased over time (P=0.03). There was an interaction between diet and time (P=0.01) in which deficient diets had the highest fusion index (FI) in both timepoints. Satellite cells from adequate pigs at 24 hours had the lowest FI, but at d3 it was not

different from deficient or excess treatments. Excess pigs were intermediate between adequate and excess diets at 24 hours, but at 72 hour had the lowest FI. The differences in satellite cell activity may be due to post-transcriptional regulation with micro-RNAs (miRNA). Expression of miR-133b, and 206 were not different between diets in differentiating satellite cells. However, the differentiation promoting miR-1 was expressed 18% more in adequate pigs than excess pigs, with deficient pigs being intermediate. Excess had the lowest fusion index and similarly the lowest expression of miR-1. The proliferation promoting miR-133a was interactively influenced by diet and time (P=0.03) in which diets deficient in energy at 72 hours had the highest expression of miR-133a over all other treatments - with the exception of excess diets at 72 hours which was not different from deficient 72 hours and all other treatment time combinations. While growth rates and final weight were similar between treatments, diet may impact growth potential later in life. Muscle fiber CSA as well as satellite cell differentiation were influenced by diet, both of which are important contributors to muscle growth.

Introduction

Growing organisms exposed to unfavorable conditions adapt to their circumstances, however, these adaptive changes during critical periods of growth can lead to adverse, permanent changes (Barker, 2007). Neonatal piglets are generally born with low energy reserves and poor insulative protection, which lead to high mortality (Curtis, 1974). Nutrient restriction can lead to the development of a "thrifty" phenotype which later alters body composition, growth rates, and insulin sensitivity (Gluckman et al., 2008). One such critical time is the neonatal period. The majority of growth in the early neonate is due to skeletal muscle hypertrophy, and during this period skeletal muscle growth occurs at a faster rate than other tissues (Davis et al., 1989; Davis et al., 1996).

Energy status can be altered through changes in energy expenditure and energy intake (Dauncey and Ingham, 1990). Postnatal skeletal muscle is impacted by both and changes due to energy status can result in lasting impacts on muscle function and growth. Skeletal muscle comprises 50% of body mass and close to 25% of basal metabolic rate. Altering the metabolic properties of muscle during the neonatal phase is associated with early muscle growth and myofiber maturation (Henriksson, 1990; Lefaucheur et al., 1998). Increasing metabolizable energy intake leads to increases in muscle net protein gain (Oddy, 1993), and nitrogen retention in neonatal pigs is linearly related to energy intake (Campbell and Dunkin, 1982). However, nutrient restriction leads to a decrease in expression of myogenic regulatory factors and muscle size. Nutrient restriction also reduces satellite cell number and activity. The population of satellite cells is greatest during the early neonatal period. Nutritional restriction during this period can lead to low weaning weights which is correlated to reductions in lean tissue growth.

Fractional growth and protein deposition occur at the highest rate during the neonatal period, and muscle grows faster than other tissues during this time (Davis et al., 1989; Davis et al., 1996). In order to support the high demand for muscle growth during the neonatal period, satellite cell incorporation provides increased capacity for protein accretion. Alterations to satellite cell proliferation and differentiation can lead to a reduction in muscle growth potential. Highly coordinated gene expression patterns progress satellite cells through the myogenic lineage to fuse with existing fibers. In order to refine the synchronization of gene expression, microRNA's (miRNA) function to inhibit translation and/or target mRNAs for degradation (Hamilton and Baulcombe, 1999; Reinhart et al., 2000; Krol et al., 2004). A group of miRNAs expressed in muscle called myoMIRs are highly involved in the myogenic process.

Sufficient energy is necessary for piglet survival and growth during the postnatal period due to their lack of adipose tissue and high energy demands to maintain body temperature and locomotion. Optimizing growth early in life is important to maximize the growth potential of the animal. Nutrient restriction early in life can lead to negative consequences to muscle growth, while high nutrient intake during the neonatal period can accelerate growth and maturity of skeletal muscle (Hu et al., 2018). In this study we hypothesized that different levels of dietary gross energy would impact muscle growth through satellite cell activity in neonatal pigs.

Methods

Animals

All pig protocols were approved by University of Maryland Animal Care and Use Committee. Thirty 1-day-old female commercial pigs (24±6h old, 1.34±0.1 kg initial body weight) were individually housed and assigned to 1 of 3 treatments based on their body weight and litter information. All pigs received an isonitrogenous diet with different energy levels with consistent

ratio of energy from carbohydrate:fat among diets. Piglets received milk replacer that varied in their energy level: 25% deficient, adequate, or 25% excess. The energy level in the adequate group was based on sow milk composition and an extrapolation of the NRC requirement for older pigs (NRC, 2012). The liquid milk replacer was reconstituted with distilled and deionized water (175g dry milk replacer with or without lactose supplementation to 1kg of liquid milk replacer) provided 8 times/day (8am - 11pm). All piglets received the same amount of milk replacer, which was designed to allow a similar growth rate to that seen with sow-rearing. The amount of feed provided daily was designed to mimic the normal growth rate under sow-reared conditions. Pigs were on trial for 16 days. Pigs were euthanized using a penetrating captive bolt gun followed by complete exsanguination. Longissimus dorsi (LD) muscle was collected for satellite cell isolation, gene expression, protein expression, and cross-sectional area (CSA) measurements.

Muscle Morphology

In order to evaluate muscle fiber CSA, LD muscle was sectioned at 10 μm thickness and stained with 5.0 μg/mL Wheat Germ Agglutinin Alexa Fluor™ 488 (Invitrogen, Carlsbad, CA) and cross stained for nuclei with 4′,6-diamidino-2-phenylindole (DAPI). Cross-sectional area of the LD was determined by tracing the loin at the 12th rib. Images of the LD and muscle fibers were analyzed for cross-sectional area using ImageJ (Rasband, 1997).

Satellite Cell Isolation

Satellite cells were isolated (Alexander et al., 2012) and cultured (Chen et al., 2017) from individual pigs as previously described and were verified to be >95% Pax7+. Cryopreserved SCs from adequate treatment pigs were cultured in growth media containing DMEM + 10% Serum + antibiotics. The serum used in the media were serum collected from each of the pigs at days 11 and 17 and pooled according to treatment. Cells were grown at 37°C in a humidified environment

containing 5% CO_2 in 15-cm plates coated with ECL Cell Attachment Matrix (MilliporeSigma, Burlington, MA) with complete media changes every other day until 80% confluence. Cells were then trypsinized and replated at a density of 3×10^3 cells/cm^2 for evaluation of proliferation and at a density of 2.5×10^4 cells/cm^2 for determination of differentiation and miRNA analysis.

Satellite Cell Proliferation and Differentiation

For analysis of *in vitro* differentiation SCs were cultured in differentiation media (DMEM + 2% serum + antibiotics) for 3 days. At the end of differentiation cells were subjected to immunocytochemical staining for myosin heavy chain. Briefly, cells were fixed with 4% paraformaldehyde for 15 min and washed twice with ice-cold PBS. The samples were then incubated for 10 min with PBS containing 0.25% Triton X-100 (PBST) and washed in PBS 3 times for 5 min. Cells were blocked in 1% BSA in PBST, and incubated with mf20 antibody (Developmental Studies Hybridoma Bank) overnight at 4° C. On the second day, samples were washed in PBS 3 times for 5 min, incubated with Dylight 488 second antibody (Thermo Fisher Scientific) for 1h, and washed in PBS 3 times for 5 minutes. Nuclei were stained with 4′,6-diamidino-2- phenylindole (DAPI; Sigma-Aldrich) in PBS. Pictures were taken at 20× magnification. The total number of nuclei were counted, and the fusion index was presented as a ratio of nuclei within myotubes containing 2+ nuclei compared with the total nuclei. In vitro satellite cell proliferation was determined at 0, 24, 48, and 72 h using the CyQUANT NF Cell Proliferation Assay Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions.

Gene Expression Analysis

Analysis of gene expression was performed as described previously (Rhoads et al., 2013). Briefly, total RNA was isolated from ~50 mg of *longissimus dorsi* tissue using TRIzol reagent

(Invitrogen, Waltham, MA). Micro RNA was isolated from ~50 mg of *longissimus dorsi* tissue and satellite cells at 24 hours and 72 hours of differentiation using the mirVana™ miRNA Isolation Kit (Life Technologies, Carlsbad, CA) according to manufacturer's instructions. Quantity and quality of RNA was verified using the Experion System (Bio-Rad Laboratories, Hercules, CA). All RNA had an RNA integrity number above 8. The eluted miRNA and RNA were diluted to 1,000ng and were reverse transcribed with iScript reverse transcriptase (Bio-Rad Laboratories, Hercules, CA). Micro RNA were reverse-transcribed using the stem loop method previously described by Kramer in 2011 by including a stem-loop primer in the reaction mix. Traditional PCR requires a template that is at least two times the length of the primers. Since miRNA's are 17-24 nucleotides in length, a stem loop primer was provided to bind to miRNA and increase their length to allow polymerase to bind and replicate. Real-time SYBR green PCR assays were performed with RNA and miRNA-specific primers (Tables 4.1 and 4.2). PCR reactions were performed in a 20µl volume using SsoAdvanced universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). Reactions contained a 1-1 primer set and diluted cDNA (10 ng). Each sample was quantified in triplicate. SYBR Green fluorescence was quantified with the CFX96 Touch Real Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). A total of 40 PCR cycles were run, and a dissociation curve was included to verify the amplification of a single PCR product. Muscle tissue relative mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method with the average of TOP2B and API5 abundance used for normalization. A standard curve was generated with oligonucleotides of the mature miRNAs measured to quantify miRNA abundance. All primer sets were evaluated for adequate efficiency before use in this study.

Statistical Analysis

Statistical analysis was performed in SAS (version 9.4; SAS Institute, Inc.). The normality for all data was tested using the Proc Univariate procedure of SAS. Homogeneity of variance was evaluated by Levene's test. Body weight, average daily gain (ADG), feed conversion ratio (FCR), and CSA area were analyzed by Proc mixed procedure with dietary treatment as the main effect. Tukey's test was used for multiple comparison tests. Proc mixed was used to analyze satellite cell proliferation and differentiation as repeated measures, with day as the repeated effect. The compound symmetry, unstructured, toeplitz, variance components, and autoregressive 1 and heterogeneous versions of covariance structures were tested and the most appropriate (lowest Akaike's information criterion, Akaike's information criterion with correction, and Bayesian information criterion values) was used for each analysis. Model included treatment, day, and interactions. Differences were considered significant when probability level $P<0.05$.

Real time PCR data for total RNA were analyzed using the $2^{-\Delta\Delta CT}$ method after normalization with the geometric mean of TOP2B and API5 expression. Gene expression was normalized back to the adequate treatment group for tissue gene expression and adequate treatment on 24 hours for satellite cell miRNA gene expression. The relative quantity ($2^{-\Delta\Delta CT}$) values were subjected to ANOVA analysis. Gene expression as analyzed by Proc mixed procedure with different diet treatments as the main effect. Tukey's test was used for multiple comparisons.

Results

Growth Parameters

Energy in important for metabolism and growth. Initial body weight did not differ between treatments ($P=0.98$; Deficient: 1.36±0.09; Adequate: 1.34±0.09; Excess: 1.33±0.09.) Final body

weight (Table 4.3) did not differ between treatments (P=0.68; Deficient: 4.46±0.15; Adequate: 4.80±0.17; Excess: 4.36±0.18). Average daily gain (ADG) did not differ between treatments (P=0.34; Deficient 0.19±0.01; Adequate 0.21±0.01; Excess 0.21±0.01). Feed efficiency calculated by weight gain (kg)/feed consumed (liquid, g) was different between treatments with excess pigs having the highest feed efficiency and deficient pigs having the lowest (P<0.001; Deficient: 0.73±0.04; Adequate: 0.89±0.04; Excess: 1.13±0.04).

Cross-Sectional Area

High energy intake has been shown to improve muscle growth and maturity in neonatal pigs (Hu et al., 2018). In this study, we evaluated LD muscle cross-sectional area (CSA) and fiber CSA (Figure 4.1). There were no differences in either LD muscle CSA or LD fiber CSA between treatments.

Satellite Cells

Satellite cell proliferation was not different between dietary treatment, however, as expected satellite cell proliferation increased over time (P=0.03) (Figure 4.2). Fusion index was interactively influenced by diet and time, in which deficient diets at 72 hours had the highest fusion index, while adequate at 24 hours had the lowest with all other treatment by time combinations being intermediate between the two (P=0.01) (Figure 4.3).

Gene Expression

This study evaluated the expression of Insulin-like Growth Factor-1 (IGF1), Myogenic Differentiation 1 (MyoD), Myogenin (MyoG), Myocyte Enhancer Factor 2A (MEF2A), 5' adenosine monophosphate-activated protein kinase (AMPK), Myostatin, Eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1), Citrate Synthase (CS), Succinate Dehydrogenase (SDH), Paired Box 7 (Pax7) and Myosin Heavy Chain (MyHC) isoforms. Dietary energy intake

did not influence the expression of IGF1, MyoD, MyoG, MEF2A, AMPK, myostatin, 4EBP1, AMPK, or CS (Figures 4.4 and 4.5). Deficient diets had a higher expression of SDH compared to adequate and deficient diets ($P<0.01$). Deficient diets also exhibited a greater expression ($P=0.04$) of Pax7 than adequate and deficient diets. Energy intake has been shown to influence skeletal muscle fiber type. No differences in Myosin Heavy Chain (MyHC)-I, IIA, IIB between dietary treatments were found (Figure 4.6). Diets consisting of excess and deficient energy had a lower expression of MyHC-IIX than adequate diets ($P=0.03$).

Micro-RNA expression

Micro-RNAs play an important role in the progression of satellite cells through the myogenic lineage. In this study, diet did not influence the expression of miR-133b and miR-206 (Figure 4.7). However, miR-1 expression was increased in deficient diets compared to excess diets ($P=0.02$). Furthermore, the expression of miR-1 increased with time during differentiation. The expression of miR-133a was interactively influenced by diet and time in which deficient diets at 72 hours of had the highest expression compared to other treatment by time combinations, with the exception of excess diets at 72 hours, which was intermediate between deficient 72 hours and other diet by time combinations.

Discussion

Undernutrition during the neonatal period can have significant consequences on animal health and growth. Because piglets lack fat deposits or brown fat, piglets need a sufficient supply of energy to maintain thermoregulation and locomotion (Pastorelli et al., 2009). Energy is the first limiting factor in young pigs and is required for maintenance and deposition of protein and fat. It is not well understood how energy levels in the neonatal pig influence growth and muscle development. Previous work found that muscle growth is more affected by undernutrition than

body weight gain (Lefaucheur et al., 1998). Restricted nutrition during the postnatal period leads to a 12% reduction in weaning weight (Beaulieu et al., 2010; Douglas et al., 2014). In this study, gross energy was manipulated through lactose and fat and content in the diet. The utilization of metabolizable energy from fat is more efficient than the metabolizable energy from protein, starch, or fiber (Just, 1982; Just et al., 1983; Noblet and Perez, 1993). Low energy intake can have lasting effects on body composition, as previous studies report that piglets weighing 15kg fed a 40% deficient energy diet until reaching 25kg had reduced protein content and increased fat at slaughter (Skiba et al., 2001). In the current study, feed conversion ratio did improve with increasing energy levels. Providing 25% excess dietary energy to neonatal pigs did not improve growth rate or final body weight. Conversely, feeding a 25% deficient energy diet was not detrimental to growth rate of final body weight, indicating that the recommended dietary energy is sufficient to sustain growth. However, it may also indicate that the levels fed were not extreme enough to produce a response.

Pig skeletal muscle consists of four different fiber types (I, IIA, IIX, IIB) (Lefaucheur et al., 1998). These fiber types are classified by their myofibril ATPase, oxidative capacity, color, and size. Skeletal muscle oxidative capacity, which varies by fiber types depending on differences in energy demands and reliance on mitochondrial oxidative phosphorylation activities, for instance type I > type 2A>type 2X>type 2B (Martin et al., 1985; Old and Johnson, 1989; Bloemberg and Quadrilatero, 2012). While total number of muscle fibers is not affected by undernutrition, fiber type shifts and differences in hypertrophy are observed. Fiber type transitions occur in an obligatory pathway for that orders I > IIa> IIx > IIb (Pette and Staron, 2000). In pigs, undernutrition between 3-7 weeks causes an increase in the proportion of slow type 1 fibers in the rhomboideus while the longissimus was not affected (Harrison et al., 1996). Consistently, this

study did not observe any differences in type I or IIA expression between dietary treatments in the *longissimus dorsi* muscle.

Undernutrition leads to reduced hypertrophy in IIa fibers (Lefaucheur et al., 1998). Conflicting studies have reported that undernutrition leads to a selective preservation of the CSA of type I and IIA fibers as they are more resilient to energy deficiency, while others report no differences between the reduction of the CSA between fiber types (Harrison et al., 1996; Lefaucheur et al., 1998). The conflicting reports may be due to differences in the age or severity of the deficiency, indicating that muscle fibers in early postnatal period are more susceptible to energy deficiencies than weaning age. In this study, no differences in fiber CSA found CSA between treatments, however, the CSA of specific fiber types was not measured. Furthermore, no differences were observed in MyHC expression with the exception of type IIX. Adequate energy treatment had the greatest expression of MyHC IIX. Muscle fibers are responsive and capable of adapting to environment, growth factors, and functional demands. While expression may be different, fiber type transitions require time to remove myosin heads and replace with the new form. Therefore, MyHC expression is not indicative of fiber type, but can provide insight into the transitions. Deficient diets may have greater oxidative fibers, while excess energy may have more glycolytic IIB fibers.

Oxidative potential is often measured by CS activity because of its role as a key regulator for aerobic energy production (Chaudhary et al., 1992). Citrate synthase activity is most closely related to type IIA and type I fibers (Delp and Duan, 1996). In this study, CS was not affected by dietary energy levels, which is consistent with previous studies indicating that undernutrition did not influence overall oxidative capacity (Lefaucheur et al., 1998). Moreover, undernutrition decreased muscle protein concentration and selectively reduced the CSA of the future fast-twitch

glycolytic fibers in *longissimus lumborum* muscle. Another marker for skeletal muscle oxidative capacity is SDH due to its role in regulating metabolism-related genes and promotion of glucose and lipid metabolism. In the current study, SDH was upregulated in deficient diets, which may indicate that deficient diets are needing to upregulate genes involved in glucose and lipid metabolism through increased SDH. Activation of AMPK signifies low energy balance in the cell, and helps to maintain energy stores by refining anabolic and catabolic pathways (Hardie et al., 2012). However, in this study, no differences were found in AMPK expression level. Translation initiation is regulated by the phosphorylation of 4EBP1 through its binding to eIF4E in response to changes in nutrients and growth factors (Haghighat et al., 1995; Khaleghpour et al., 1999). Growth rate is highly correlated with IGF-I serum concentrations (Lund-Larsen and Bakke, 1975; Whang et al., 2003; CHO et al., 2008). In this study, no differences were observed in either growth rate, or IGF-I concentrations.

During the process of stem cell commitment to the myogenic lineage, miRNAs function to facilitate and refine the complex expression pattern of myogenic genes through inhibition of translation or marking target mRNAs for degradation. Differentiation is promoted through miR-1/206. Micro-RNA -1 and -206 differ from one another by one nucleotide that resides outside of the seed sequence, therefore, the two miRNAs share many of the same targets (Dey et al., 2011). One such target is histone deacetylase 4 (HDAC4), a transcriptional repressor of myogenic genes, mainly inhibiting the transcription factor MEF2A and- C, which are important in differentiation and muscle hypertrophy (Lu et al., 2000; Ikeda et al., 2009). In this study, there were no differences in expression level of MEF2A in muscle tissue, however that does not indicate that miR-1 is not playing a role in its translation.

In this study, dietary energy level did not influence the expression of miR-206 in satellite cells. Deficient diets had the greatest expression of the differentiation promoting miR-1 and, correspondingly, the greatest fusion index. Excess diets had the lowest expression of miR-1 and the lowest fusion index. While at 24 hours, adequate dietary treatment had the lowest fusion rate, at 72 hours it surpassed the excess diet fusion rate, which corresponded with an increase in miR-1 expression at 72 hours in adequate treatment.

Another target of miRNA-1/206 is the satellite cell marker, Pax7. Satellite cells are characterized by their expression of Pax7, which is required for maintaining quiescence, proliferation, and inhibits the precocious differentiation. Down regulation of Pax7 allows for progression of the myogenic lineage (Buckingham, 2007; Kuang et al., 2007). While Pax7 expression was highest in deficient diets, the increased abundance of miR-1 in satellite cells may be functioning to repress translation. The increase in Pax7 in deficient diets could also be indicative of a greater number of satellite cells present in the muscle tissue due to enhanced proliferation. However, proliferation rates were not different between treatments, which may indicate diminished function due to deficient energy.

Conclusion

Our data indicate that deficient energy during the postnatal period does not lead to changes in growth rate but may be detrimental to satellite cell function and therefore growth potential. Providing excess energy did improve feed efficiency, however, did not enhance growth or satellite cell activity, indicating that the level of energy provided to neonatal pigs is sufficient for proper growth. These data have practical implications as alterations to the behavior of satellite cells can have sustained impacts on growth and composition. This highlights that sufficient energy should be provided to maintain satellite cell function in neonatal pigs.

Acknowledgments

This study was funded in part by USDA (2015-67015-25094).

References

Alexander, L. S., B. S. Seabolt, R. P. Rhoads, and C. H. Stahl. 2012. Neonatal phosphate nutrition alters in vivo and in vitro satellite cell activity in pigs. Nutrients 4(6):436-448. doi: 10.3390/nu4060436

Barker, D. J. P. 2007. The origins of the developmental origins theory. Journal of Internal Medicine 261(5):412-417. doi: 10.1111/j.1365-2796.2007.01809.x

Beaulieu, A. D., J. L. Aalhus, N. H. Williams, and J. F. Patience. 2010. Impact of piglet birth weight, birth order, and litter size on subsequent growth performance, carcass quality, muscle composition, and eating quality of pork1. Journal of Animal Science 88(8):2767-2778. doi: 10.2527/jas.2009-2222

Bloemberg, D., and J. Quadrilatero. 2012. Rapid determination of myosin heavy chain expression in rat, mouse, and human skeletal muscle using multicolor immunofluorescence analysis. PLoS One 7(4):e35273. doi: 10.1371/journal.pone.0035273

Buckingham, M. 2007. Skeletal muscle progenitor cells and the role of Pax genes. Comptes rendus biologies 330(6-7):530-533.

Campbell, R., and A. Dunkin. 1982. The effects of birth weight and level of feeding in early life on growth and development of muscle and adipose tissue in the young pig. Animal Science 35(2):185-192. doi: 10.1017/S0003356100027343

Chaudhary, R., B. P. Chowdhary, I. Harbitz, I. Gustavsson, and C. T. Evans. 1992. Localization of the citrate synthase (CS) gene to the p12-p13 bands of chromosome 5 in pigs by in situ hybridization. Hereditas 117(1):39-43. doi: 10.1111/j.1601-5223.1992.tb00005.x

Chen, Y., H. Zhu, S. R. McCauley, L. Zhao, S. E. Johnson, R. P. Rhoads, and S. W. El-Kadi. 2017. Diminished satellite cell fusion and S6K1 expression in myotubes derived from skeletal muscle of low birth weight neonatal pigs. Physiological Reports 5(3):e13075. doi: 10.14814/phy2.13075

CHO, J. H., Y. J. CHEN, B. J. MIN, J. S. YOO, Y. WANG, and I. H. KIM. 2008. Effects of reducing dietary crude protein on growth performance, odor gas emission from manure and blood urea nitrogen and IGF-1 concentrations of serum in nursery pigs. Animal Science Journal 79(4):453-459. doi: 10.1111/j.1740-0929.2008.00549.x

Curtis, S. E. 1974. Responses of the piglet to perinatal stressors. Journal of Animal Science 38(5):1031-1036.

Dauncey, M. J., and D. L. Ingham. 1990. Respiratory enzymes in muscle: Interaction between environmental temperature, nutrition and growth. Journal of Thermal Biology 15(3):325-328. doi: 10.1016/0306-4565(90)90021-9

Davis, T. A., D. G. Burrin, M. L. Fiorotto, and H. V. Nguyen. 1996. Protein synthesis in skeletal muscle and jejunum is more responsive to feeding in 7-than in 26-day-old pigs. American Journal of Physiology-Endocrinology and Metabolism 270(5):E802-E809. doi: 10.1152/ajpendo.1996.270.5.E802

Davis, T. A., M. L. Fiorotto, H. V. Nguyen, and P. J. Reeds. 1989. Protein turnover in skeletal muscle of suckling rats. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 257(5):R1141-R1146. doi: 10.1152/ajpregu.1989.257.5.R1141

Delp, M. D., and C. Duan. 1996. Composition and size of type I, IIA, IID/X, and IIB fibers and citrate synthase activity of rat muscle. Journal of Applied Physiology 80(1):261-270. doi: 10.1152/jappl.1996.80.1.261

Dey, B. K., J. Gagan, and A. Dutta. 2011. miR-206 and-486 induce myoblast differentiation by downregulating Pax7. Molecular and cellular biology 31(1):203-214.
Douglas, S. L., S. A. Edwards, and I. Kyriazakis. 2014. Management strategies to improve the performance of low birth weight pigs to weaning and their long-term consequences1,2. Journal of Animal Science 92(5):2280-2288. doi: 10.2527/jas.2013-7388
Gluckman, P. D., M. A. Hanson, C. Cooper, and K. L. Thornburg. 2008. Effect of In Utero and Early-Life Conditions on Adult Health and Disease. New England Journal of Medicine 359(1):61-73. doi: 10.1056/NEJMra0708473
Haghighat, A., S. Mader, A. Pause, and N. Sonenberg. 1995. Repression of cap-dependent translation by 4E-binding protein 1: competition with p220 for binding to eukaryotic initiation factor-4E. The EMBO Journal 14(22):5701-5709. doi: 10.1002/j.1460-2075.1995.tb00257.x
Hamilton, A. J., and D. C. Baulcombe. 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286(5441):950-952.
Hardie, D. G., F. A. Ross, and S. A. Hawley. 2012. AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nature Reviews Molecular Cell Biology 13(4):251-262. doi: 10.1038/nrm3311
Harrison, A. P., A. M. Rowlerson, and M. J. Dauncey. 1996. Selective regulation of myofiber differentiation by energy status during postnatal development. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 270(3):R667-R674. doi: 10.1152/ajpregu.1996.270.3.R667
Henriksson, J. 1990. The possible role of skeletal muscle in the adaptation to periods of energy deficiency. European Journal of Clinical Nutrition 44(Supplement 1):55-64.
Hu, L., F. Han, L. Chen, X. Peng, D. Chen, D. Wu, L. Che, and K. Zhang. 2018. High nutrient intake during the early postnatal period accelerates skeletal muscle fiber growth and maturity in intrauterine growth-restricted pigs. Genes & Nutrition 13(1):23. doi: 10.1186/s12263-018-0612-8
Ikeda, S., A. He, S. W. Kong, J. Lu, R. Bejar, N. Bodyak, K.-H. Lee, Q. Ma, P. M. Kang, T. R. Golub, and W. T. Pu. 2009. MicroRNA-1 negatively regulates expression of the hypertrophy-associated calmodulin and Mef2a genes. Molecular and cellular biology 29(8):2193-2204. doi: 10.1128/MCB.01222-08
Just, A. 1982. The net energy value of balanced diets for growing pigs. Livestock Production Science 8(6):541-555. doi: 10.1016/0301-6226(82)90032-X
Just, A., J. Fernández, and H. Jørgensen. 1983. The net energy value of diets for growth in pigs in relation to the fermentative processes in the digestive tract and the site of absorption of the nutrients. Livestock Production Science 10(2):171-186. doi: 10.1016/0301-6226(83)90033-7
Khaleghpour, K., S. Pyronnet, A.-C. Gingras, and N. Sonenberg. 1999. Translational Homeostasis: Eukaryotic Translation Initiation Factor 4E Control of 4E-Binding Protein 1 and p70 S6 Kinase Activities. Molecular and Cellular Biology 19(6):4302-4310. doi: 10.1128/mcb.19.6.4302
Kramer, M. F. 2011. Stem-loop RT-qPCR for miRNAs. Current protocols in molecular biology 95(1):15.10. 11-15.10. 15. doi: 10.1002/0471142727.mb1510s95
Krol, J., K. Sobczak, U. Wilczynska, M. Drath, A. Jasinska, D. Kaczynska, and W. J. Krzyzosiak. 2004. Structural features of microRNA (miRNA) precursors and their

relevance to miRNA biogenesis and small interfering RNA/short hairpin RNA design. Journal of Biological Chemistry 279(40):42230-42239.
Kuang, S., K. Kuroda, F. Le Grand, and M. A. Rudnicki. 2007. Asymmetric Self-Renewal and Commitment of Satellite Stem Cells in Muscle. Cell 129(5):999-1010. doi: 10.1016/j.cell.2007.03.044
Lefaucheur, L., R. Hoffman, D. Gerrard, C. Okamura, N. Rubinstein, and A. Kelly. 1998. Evidence for three adult fast myosin heavy chain isoforms in type II skeletal muscle fibers in pigs. Journal of animal science 76(6):1584-1593. doi: 10.2527/1998.7661584x
Lu, J., T. A. McKinsey, C.-L. Zhang, and E. N. Olson. 2000. Regulation of skeletal myogenesis by association of the MEF2 transcription factor with class II histone deacetylases. Molecular cell 6(2):233-244.
Lund-Larsen, T. R., and H. Bakke. 1975. Growth Hormone and Somatomedin Activities in Lines of Pigs Selected for Rate of Gain and Thickness of Backfat. Acta Agriculturae Scandinavica 25(3):231-234. doi: 10.1080/00015127509435044
Martin, T. P., A. C. Vailas, J. B. Durivage, V. R. Edgerton, and K. R. Castleman. 1985. Quantitative histochemical determination of muscle enzymes: biochemical verification. J Histochem Cytochem 33(10):1053-1059. doi: 10.1177/33.10.4045183
Noblet, J., and J. M. Perez. 1993. Prediction of digestibility of nutrients and energy values of pig diets from chemical analysis1. Journal of Animal Science 71(12):3389-3398. doi: 10.2527/1993.71123389x
NRC, N. R. C. 2012. Nutrient requirements of swine. National Academies Press.
Oddy, V. 1993. Regulation of muscle protein metabolism in sheep and lambs: nutritional, endocrine and genetic aspects. Australian Journal of Agricultural Research 44(5):901-913. doi: 10.1071/AR9930901
Old, S. L., and M. A. Johnson. 1989. Methods of microphotometric assay of succinate dehydrogenase and cytochrome c oxidase activities for use on human skeletal muscle. Histochem J 21(9-10):545-555.
Pastorelli, G., M. Neil, and I. Wigren. 2009. Body composition and muscle glycogen contents of piglets of sows fed diets differing in fatty acids profile and contents. Livestock science 123(2-3):329-334. doi: 10.1016/j.livsci.2008.11.023
Pette, D., and R. S. Staron. 2000. Myosin isoforms, muscle fiber types, and transitions. Microscopy Research and Technique 50(6):500-509. doi: 10.1002/1097-0029(20000915)50:6<500::Aid-jemt7>3.0.Co;2-7
Rasband, W. S. 1997. ImageJ. Bethesda, MD.
Reinhart, B. J., F. J. Slack, M. Basson, A. E. Pasquinelli, J. C. Bettinger, A. E. Rougvie, H. R. Horvitz, and G. Ruvkun. 2000. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. nature 403(6772):901.
Rhoads, R., K. Flann, T. Cardinal, C. Rathbone, X. Liu, and R. E. Allen. 2013. Satellite cells isolated from aged or dystrophic muscle exhibit a reduced capacity to promote angiogenesis in vitro. Biochemical and biophysical research communications 440(3):399-404. doi: 10.1016/j.bbrc.2013.09.085
Skiba, G., H. Fandrejewski, S. Raj, and D. Weremko. 2001. The performance and body composition of growing pigs during protein or energy deficiency and subsequent realimentation. Journal of Animal and Feed Sciences 10(4):633-647. (journal article) doi: 10.22358/jafs/68015/2001

Whang, K. Y., S. W. Kim, S. M. Donovan, F. K. McKeith, and R. A. Easter. 2003. Effects of protein deprivation on subsequent growth performance, gain of body components, and protein requirements in growing pigs. J Anim Sci 81(3):705-716. doi: 10.2527/2003.813705x

Tables and Figures

Table 4.1 Nucleotide sequences for real-time PCR primers

Target	Direction	Sequence
API5	Forward	GCATTTTTAGTAGCATAGGCCCTTT
	Reverse	AACTTGAGGGAAGATTAACTGTGGAA
AMPKinase	Forward	ACGGAGCAAGTAACCCCAGC
	Reverse	GCCACGAGGATGACGATGAA
IGF-1	Forward	GCACATCACATCCTCTTCGC
	Reverse	GCCTCCTCAGATCACAGCTC
MEF2A	Forward	TGAATACCCAGAGGATAAGCAGTT
	Reverse	TAATCGGTGTTGTAGGCGG
MyHC I	Forward	AAGGGCTTGAACGAGGAGTAGA
	Reverse	TTATTCTGCTTCCTCCAAAGGG
MyHC IIa	Forward	GCTGAGCGAGCTGAAATCC
	Reverse	ACTGAGACACCAGAGCTTCT
MyHC IIb	Forward	ATGAAGAGGAACCACATTA
	Reverse	TTATTGCCTCAGTAGCTTG
MyHC IIx	Forward	AGAAGATCAACTGAGTGAACT
	Reverse	AGAGCTGAGAAACTAACGTG
MSTN	Forward	CCAGAGAGATGACAGCAGTGATG
	Reverse	TTCCTTCCACTTGCATTAGAAGATC
MyoD	Forward	GCGTGCAAACGCAAGACCACTAA
	Reverse	AGTCTCGAAGGCCTCGTTGACTTT
MyoG	Forward	TGACCCTACAGATGCCCACAATCT
	Reverse	GTTGGGCATGGTTTCATCTGGGAA
Pax7	Forward	CAACCACATCCGCCACAAGATAGT
	Reverse	AGAGGATCTTGGAGACACAGCCAT
SDH	Forward	TGGCCGGCACAGCTCCCAAT
	Reverse	GCACGTTCCCTGCTCTCCCG
TOP2B	Forward	AACTGGATGATGCTAATGATGCT
	Reverse	TGGAAAAACTCCGTATCTGTCTC

Table 4.2 Nucleotide sequences for miRNA real-time PCR primers

Target	Primer	Primer sequences (5'-3')
ssc-miR-1	Stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTACATA
	Forward	GGCGGCTGGAATGTAAAGAAG
	Reverse	CAGTGCAGGGTCCGAGGTAT
	Oligonucleotide	TGGAATGTAAAGAAGTATGTA
ssc-miR-133a-3p	Stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGCTG
	Forward	TTGCGTTGGTCCCCTTCA
	Reverse	CAGTGCAGGGTCCGAGGTAT
	Oligonucleotide	TTGGTCCCCTTCAACCAGCTG
ssc-miR-133b	Stem-loop	GCGTGGTCCACACCACCTGAGCCGCCACGACCACGCATAGCTGG
	Forward	CCAGCCTTTGGTCCCCTTC
	Reverse	TCCACACCACCTGAGCCG
	Oligonucleotide	TTTGGTCCCCTTCAACCAGCTAT
ssc-miR-206	Stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCACAC
	Forward	CCGCGTGGAATGTAAGGAAGT
	Reverse	CAGTGCAGGGTCCGAGGTAT
	Oligonucleotide	TGGAATGTAAGGAAGTGTGTGA

Table 4.3 Impact of excess energy levels on growth performance of neonatal pigs[1]

	Deficient	Adequate	Excess
Initial BW (kg)	1.36±0.09	1.34±0.09	1.33±0.09
Final BW (kg)	4.46±0.15	4.80±0.17	4.36±0.18
ADG (kg)[2]	0.19±0.01	0.21±0.01	0.21±0.01
FCR[3]	0.73±0.04 [c]	0.89±0.04 [b]	1.13±0.04 [a]

[1] Values are means ± SEMs. Labeled means in a row without a common letter differ, $P < 0.05$ (a > b)

[2] Average Daily Gain

[3] Feed Conversion Ratio calculated by weight gain (kg)/ feed consumed (liquid, g)

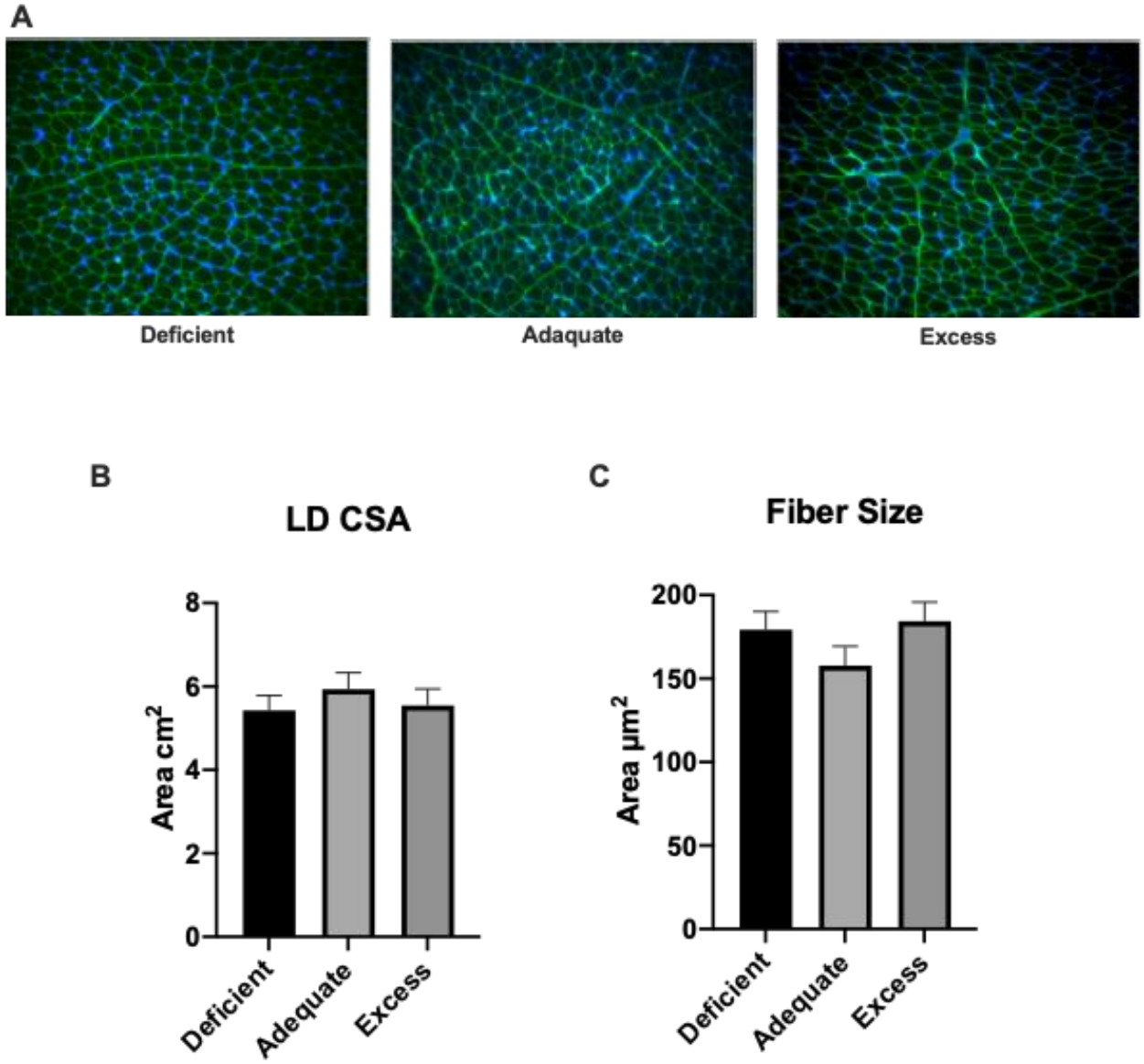

Figure 4.1 Cross sectional area (CSA) analysis of the *longissimus dorsi* muscle and fibers of pigs fed differing energy levels. Cross sectional area of muscle (B), fiber cross sectional area (C), and representative images of fiber cross sectional area (A) of pigs fed a diet containing either 25% deficient energy, adequate energy, or a 25% excess energy diet. Results are means ± SEM. Means without a common letter are different at $P<0.05$.

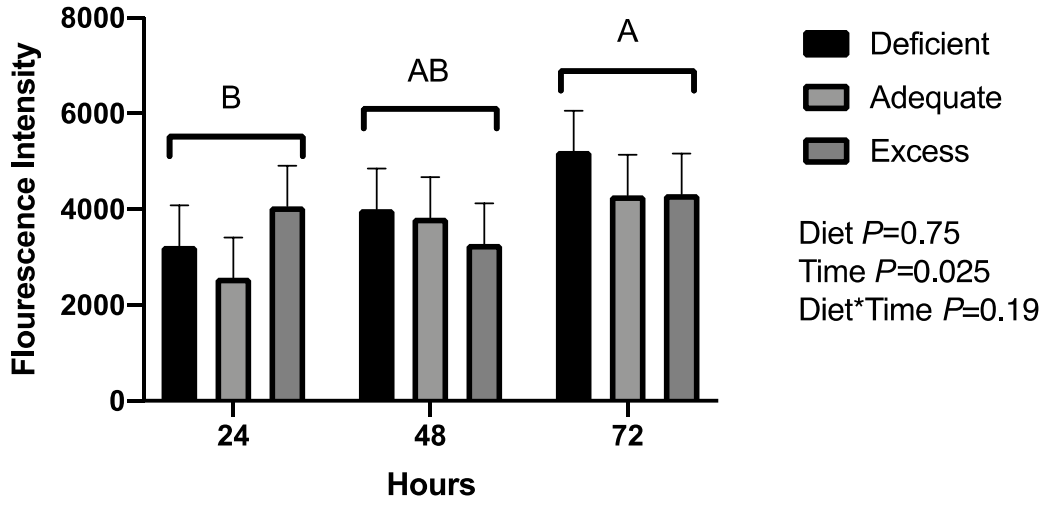

Figure 4.2 Satellite cell proliferation of pigs fed differing energy levels at 24, 48, and 72 hours. Pigs were fed a diet containing either 25% deficient energy, adequate energy, or a 25% excess energy diet at 24, 48, and 72 hours. Results are means ± SEM. Timepoints without a common letter are different at $P<0.05$.

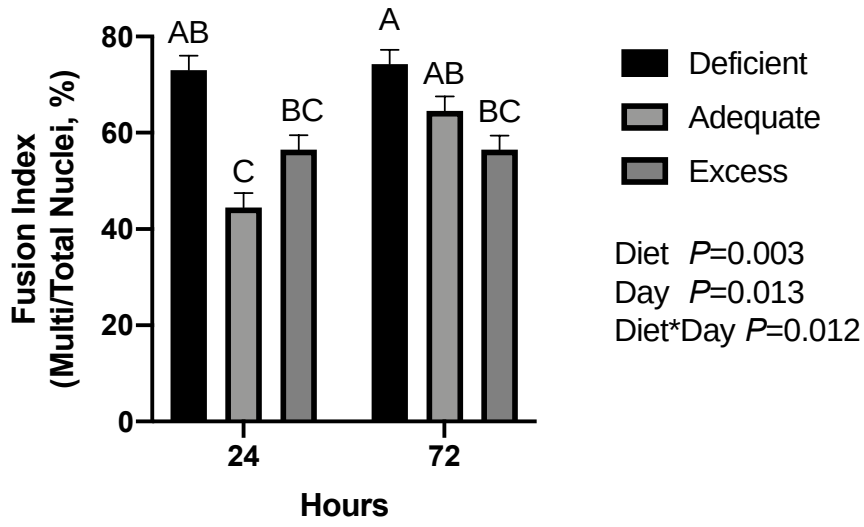

Figure 4.3 Satellite cell differentiation from the *longissimus dorsi* muscle of pigs fed varying levels of dietary energy. Fusion index is expressed as the percentage of total nuclei that are located in myotubes. Pigs were fed a diet containing either 25% deficient energy, adequate energy, or a 25% excess energy diet. Values are means ± SEMs. Means at a time without a common letter differ $P < 0.05$.

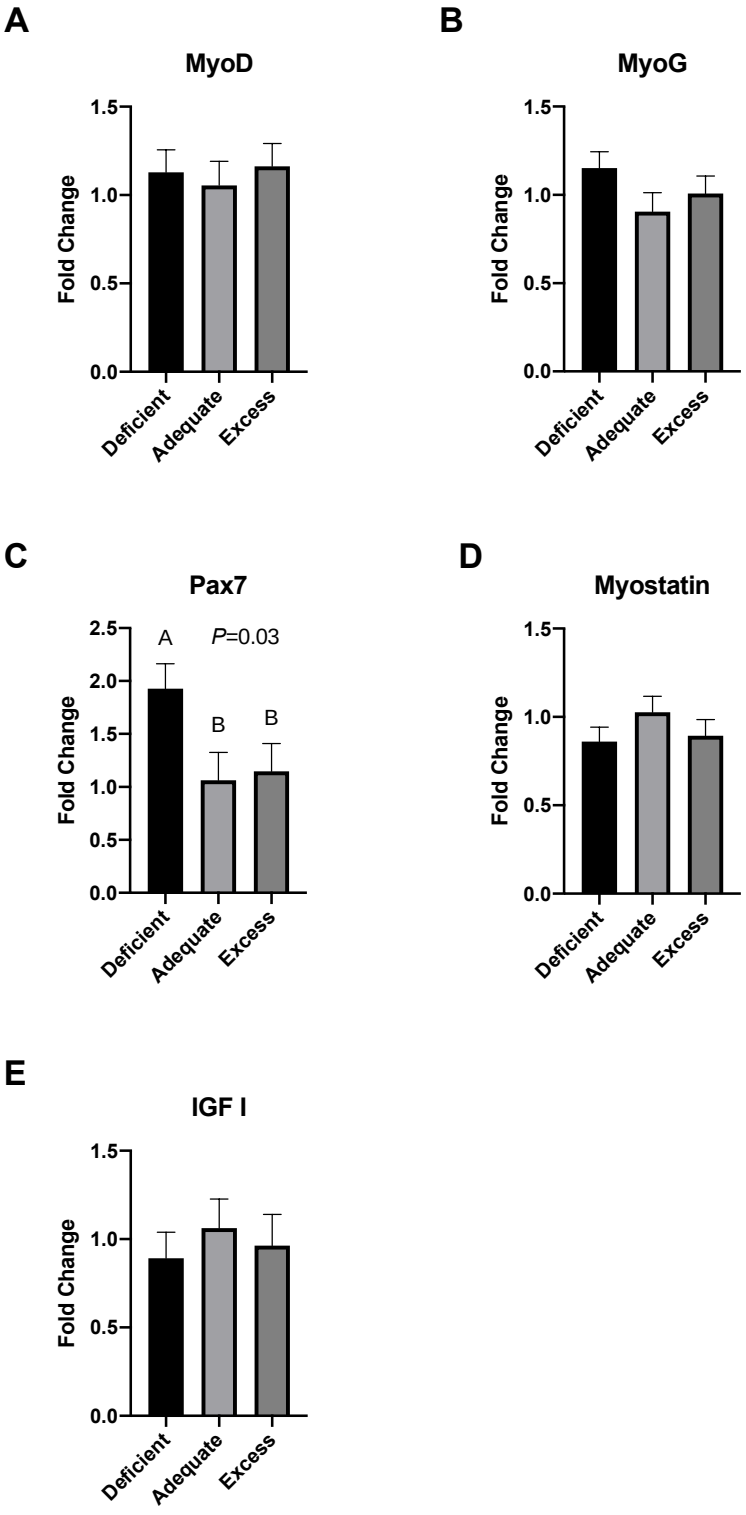

Figure 4.4 mRNA expression of genes involved in muscle growth in the *longissimus dorsi* muscle of pigs fed differing levels of dietary energy. Pigs were fed a diet containing either 25% deficient energy, adequate energy, or a 25% excess energy diet. Results are means ± SEM. Means without a common letter are different at $P<0.05$ (A>B).

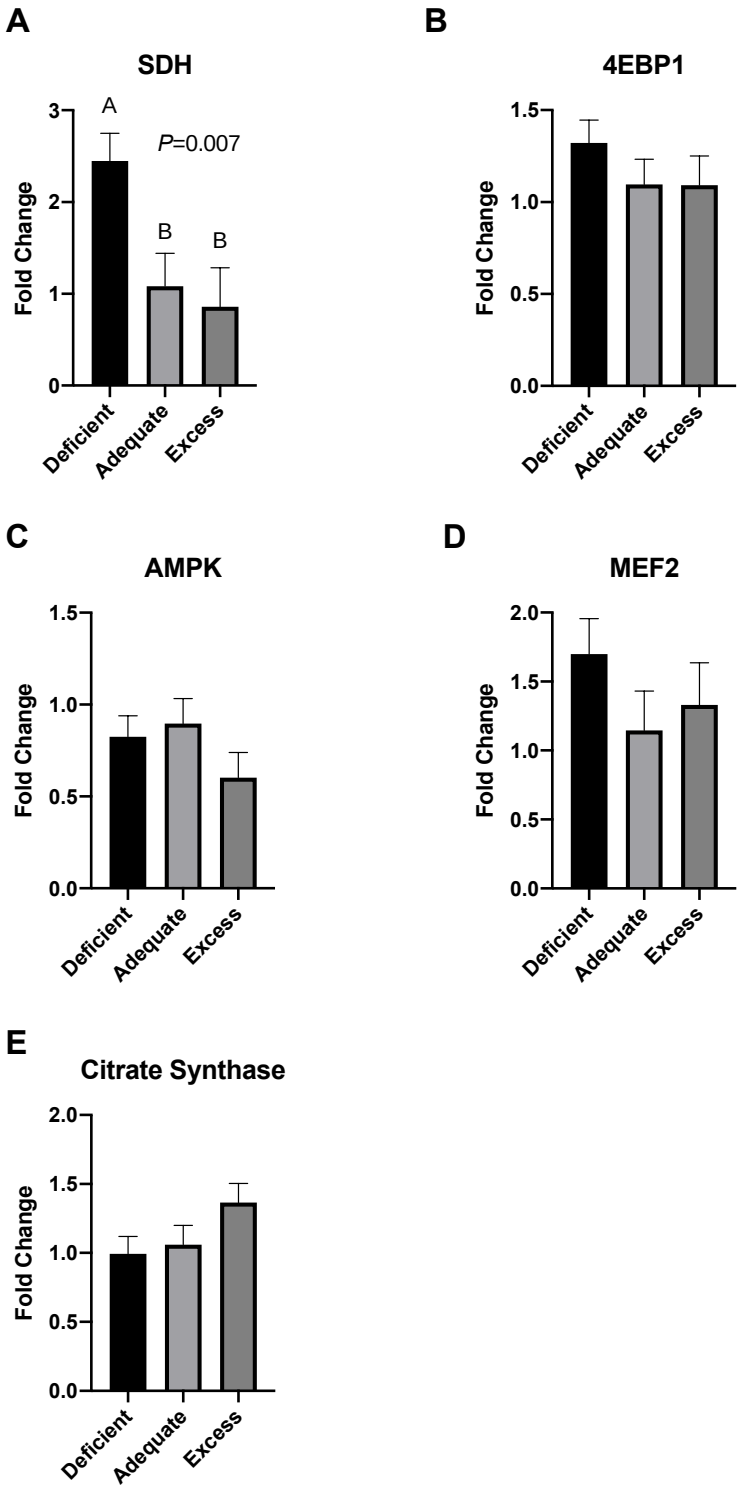

Figure 4.5 mRNA expression of genes involved in energy sensing in the *longissimus dorsi* muscle of pigs fed differing levels of dietary energy. Pigs were fed a diet containing either 25% deficient energy, adequate energy, or a 25% excess energy diet. Results are means ± SEM. Means without a common letter are different at $P<0.05$ (A>B).

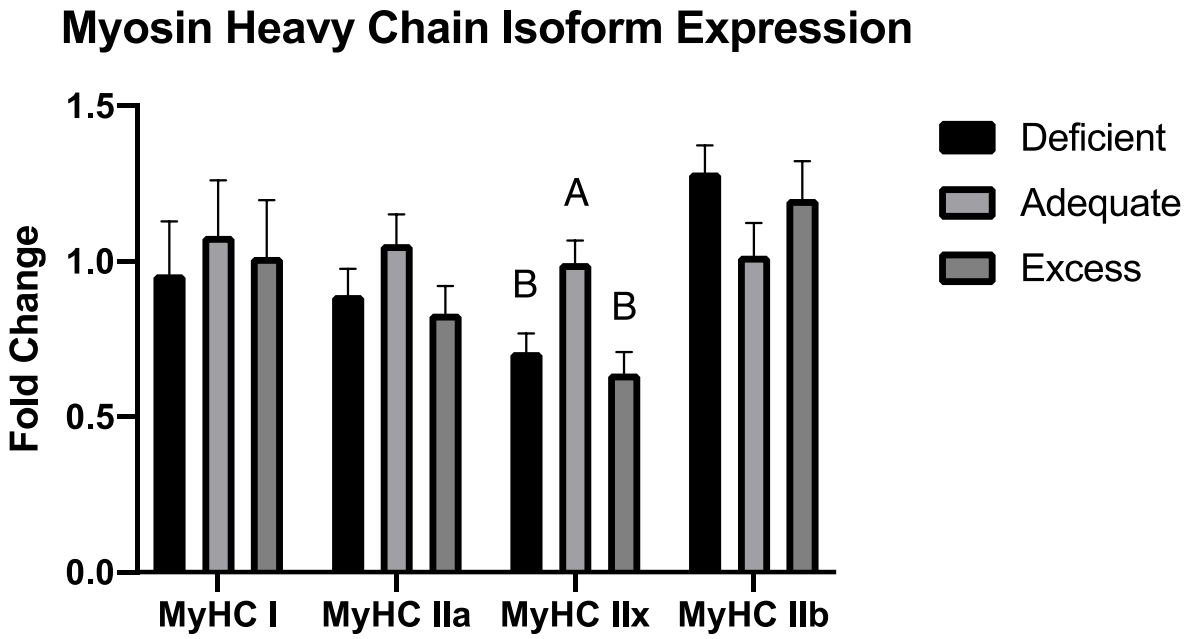

Figure 4.6 mRNA expression of the myosin heavy chain (MyHC) isoforms in the *longissimus dorsi* muscle of pigs fed differing levels of dietary energy. Pigs were fed a diet containing either 25% deficient energy, adequate energy, or a 25% excess energy diet. Results are means ± SEM. Means without a common letter are different at $P<0.05$ (A>B).

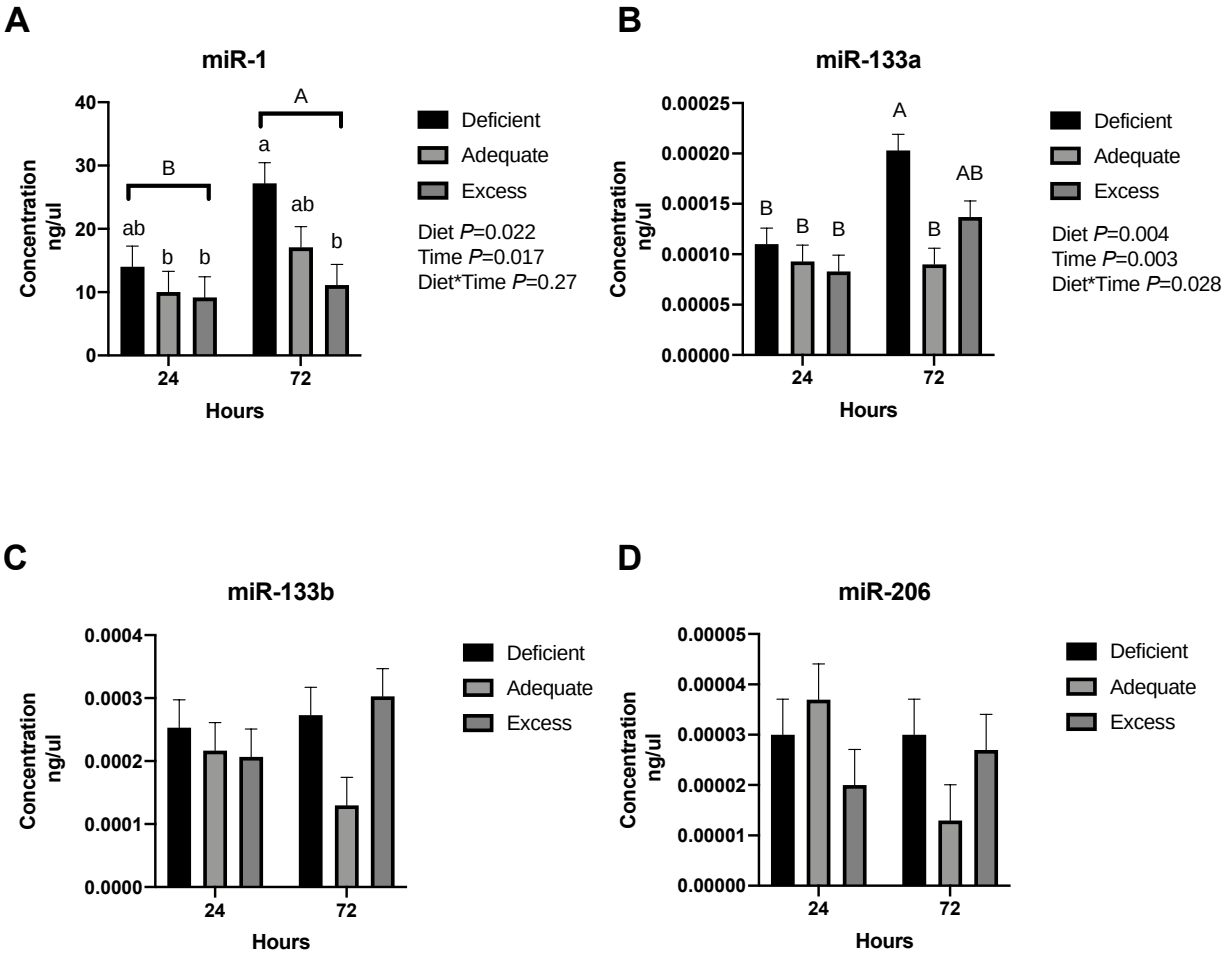

Figure 4.7 Expression of miR-1 (A), miR-133a (B), miR-133b (C), and miR-206 (D) in satellite cells of pigs fed differing levels of dietary energy. Pigs were fed a diet containing either 25% deficient energy, adequate energy, or a 25% excess energy diet at 0 hours and 72 hours of differentiation. Results are means ± SEM. Means without a common letter are different at $P<0.05$.

Chapter 5: The use of artificial sweetener and capsicum oleoresin to alleviate the negative effects of chronic heat stress on pig performance

Abstract

Substantial economic losses in animal agriculture result from animals undergoing heat stress. Pigs are especially susceptible to heat stress which results in reductions in average daily gain, altered body composition, and decreased gut function. To alleviate the negative effects of heat stress, feed additives such as capsicum oleoresin can modify inflammation and the immune response, while artificial sweeteners work to improve intestinal morphology and nutrient absorption. In this study, the supplementation of an artificial high-intensity sweetener and capsicum oleoresin (CAPS-SUC; TakTik X-Hit, Pancosma, Switzerland) to mitigate the negative effects of heat stress on pig performance was examined. Forty cross bred barrows (16.2 kg±6 kg) were assigned to one of five treatments: heat stress conditions and ad libitum feed with (HS+) or without (HS-) supplementation, thermal neutral conditions (22°C±1.2°C; 38%-73% relative humidity) with ad libitum feed (C), pair-fed to heat stress (PF), pair-fed to HS+ with supplementation (PF+). Pigs in heat stressed treatments were exposed to a cyclical environmental temperature of 12 hours at 35°C±1.2°C with 27-45% relative humidity and 12 hours at 30°C±1.1°C with 24-35% relative humidity for 21 days. Supplementation (0.1g/kg feed) began 7 days before the initiation of environmental treatment. Rectal temperatures (RT) and respiration rates (RR;breaths/minute) were recorded thrice daily, and feed intake (FI) was recorded daily. Prior to the start and at the termination of environmental treatments, a muscle biopsy of the *longissimus dorsi* was taken for metabolism analyses. Blood samples were collected every week and animals were weighed every 3 days during treatment. Pigs in HS- conditions exhibited increased RT (~0.5°C) and RR (~2 fold) compared to TN and PF groups ($P < 0.001$). Supplementation in HS+ pigs did not affect RR. Heat stress reduced FI by 12% ($P<0.001$), while supplementation did not affect feed intake. Feed efficiency (gain:feed) was not affected by heat stress, however, was

reduced by 34% in PF- animals (P<0.02). Supplementation tended to increase feed efficiency (P=0.0833). Average daily gain (ADG) was decreased 56% due to PF (P<0.001). Metabolic flexibility was retained after environmental treatment in all groups except for HS, which decreased flexibility by 8% (P=0.053). There was no change in any measure of fatty acid oxidation (complete/incomplete/total) between treatments. Citrate synthase and cytochrome C oxidase activities were lower in HS- animals after treatment, compared to control (P<0.01). Blood glucose was lower in the HS- and PF group (P=0.05). Supplementation increased monocyte number in PF+ animals (P=0.02). CAPS-SUC supplementation helped to maintain metabolic flexibility during heat stress, and improved feed efficiency and immune response during nutrient restriction.

Introduction

Elevated environmental temperatures cause high thermal loads in humans and livestock that can lead to acute, chronic, and lethal heat related disorders such as heat stress (Wilkins and Wheeler, 2004). Homeothermic animals maintain a thermal neutral core body temperature within a narrow range in which cellular processes and enzymatic reactions function optimally. Outside of this range, high temperatures may cause a breakdown in proteins and cell death (Wilkins and Wheeler, 2004). As a result of reduced growth rates during periods of heat stress, major economic losses in animal production are observed (Hahn, 1999; Marai et al., 2007; Renaudeau et al., 2011), decreased fertility (Hennessy and Williamson, 1984; Nardone et al., 2006; Hansen, 2009; Mayorga et al., 2020), as well as increased morbidity and mortality (D'Allaire et al., 1996; Bishop-Williams et al., 2015).

Heat stress responses are largely conserved across species. Respiration rate increases during hyperthermia, which is used to increase evaporative cooling through the mouth (Anderson and Bates, 1984; Patience et al., 2005). Radiant heat dissipation through the skin is assisted by a

repartitioning of blood from splanchnic organs towards peripheral blood flow (Lambert, 2009). Pigs are especially susceptible to heat stress due to the negligible amount of cooling through sweating with heat regulation occurring mainly by respiration through evaporative cooling (Ingram, 1965). Modern strains of pigs have undergone genetic selection towards increased lean mass at the cost of increased metabolic rate and elevated endogenous thermal genesis thus increasing their susceptibility for heat stress related conditions (Nienaber et al., 1997; Baumgard and Rhoads, 2013a). Consequently, reduced blood and nutrient flow compromises intestinal epithelium integrity and function (Yan et al., 2006; Pearce et al., 2013a; Pearce et al., 2013c), which leads to changes in nutrient digestibility and absorption as well as endotoxemia, hypoxia, and inflammation (Hall et al., 2001; Pearce et al., 2013b). Behavioral changes such as a reduction in locomotion and a voluntary reduction in feed intake to reduce the metabolic thermic effect. However, this reduces growth and reproductive performances impairing profitability (Nienaber et al., 1997).

Many management strategies have been implemented to alleviate the negative symptoms of heat stress in pig production. Fans and evaporative cooling systems have been used to decrease ambient temperature, while floor cooling, drip cooling, and snout cooling have been used to increase animal heat loss (McGlone et al., 1988; Silva et al., 2006). Nutritional interventions could be implemented to mitigate heat stress and are of particular interest due to the behavioral adaption of reduced feed intake during heat. Therefore, altering the composition of diets to reduce metabolic heat and increase available energy may help to reduce the heat stress response (Renaudeau et al., 2008). Alternative to modifying the composition of the diet, functional feed additives can be added to feed in a low dose to help combat environmental conditions. Functional feed additives including plant extracts, essential oils, pre- and prebiotics have been used in livestock as alternatives to

antibiotics because of their potential to improve gut health. These additives modify immune functions, improve intestinal epithelium tight junctions, and alter the gut microbiome (De Lange et al., 2010). Therefore, the use of functional feed additives may provide a cost-effective strategy to improve animal performance and gut functionality.

Supplementation with feed additives such as high-intensity artificial sweetener improve gut function. Sugars and artificial sweeteners can stimulate taste receptors in the intestinal epithelium which increases the secretion of the gut hormone glucagon-like peptide (GLP)-2 and upregulates expression of the Na+/glucose co-transporter 1 (SGLT-1) (Drucker, 1996). Both GLP-2 and SGLT-1 improve gut function and integrity as well as improve absorption. (Burrin et al., 2007; Bahrami et al., 2010; Moran et al., 2010; Shi et al., 2013; Vegge et al., 2013). Therefore, artificial sweeteners improve intestinal health and nutrient absorption, and can be applied as a potential strategy to decrease the negative impacts of heat stress.

In addition to artificial sweeteners, capsicum oleoresin, derived from hot peppers, has also been shown to improve gut function (Iwai et al., 2003; Leung, 2008). The principle component, Capsaicin, increases blood flow to tissues including gastric mucosa and provides a protective mechanism against noxious stimuli (Yonei et al., 1990). Capsicum oleoresin has anti-inflammatory properties on lymphocytes in the intestinal mucosa by modulating transcription of inflammatory genes (Kim et al., 2010; Nevius et al., 2012). When fed to weaned piglets, capsicum oleoresin improved growth performance, decreased the incidence of diarrhea and decreased the severity of immune response when challenged with *Escherichia coli* and porcine reproductive and respiratory syndrome virus (Liu et al., 2013a; Liu et al., 2013b).

The use of a combination of artificial sweeteners and capsicum oleoresin (CAP-SUC) was found to improve feed efficiency over non-supplemented pigs during a 3 day heat stress period

(Biggs et al., 2020). This increased efficiency resulted in supplemented animals gaining 0.12 kg more body weight per kilogram of feed than their non-supplemented counterparts. Increased body weight gain is mainly driven by skeletal muscle growth. As a result, skeletal muscle substrate usage can influence whole body metabolism. Metabolic flexibility is the ability to metabolize and switch between fuel substrates in order to adapt to changes in energy demands and activity (Goodpaster and Sparks, 2017). As a result of heat stress, muscle becomes less metabolically flexible and has limited use of non-esterified fatty acids (Zhao et al., 2018). The increased feed efficiency in CAP-SUC supplemented animals may be due to retained skeletal muscle metabolic flexibility.

This study sought to ascertain the effects of feeding a combination of capsicum oleoresin and a high-intensity artificial sweetener on the ability to alleviate the negative effects of a chronic period of heat stress in growing pigs. We hypothesized that supplementation would improve growth performance and metabolic flexibility of challenged pigs and mitigate the severity of the heat stress response.

Materials and Methods

Animals and Treatments

All procedures utilizing animals was approved by the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee. Forty cross bred barrows were transported to Litton-Reaves Hall at 16.2kg±6kg and individually housed in one of similarly configured rooms. Both rooms were held at a 12 hour light dark cycle where light was provided between 0800 h and 2000 h. All animals were allowed a 7 day acclimation period in which rooms were maintained in thermal neutral conditions with ad libitum access to feed commercial diet based on NRC requirements and thermal neutral conditions (22°C) (Figure 5.1). Animals (n=40) were

weighed and assigned to one of five treatments upon arrival based on average body weight. The five treatments consisted of an ad libitum feed with heat stress conditions (HS), ad libitum feed with supplementation and heat stress conditions (HS+), pair-fed to heat stress in thermal neutral conditions (PF), pair-fed to HS+ in thermal neutral conditions with supplementation (PF+), and a thermal neutral control with ad libitum access to feed (C). Pigs in thermal neutral conditions were maintained at 22°C±1.2°C with 38%-73% relative humidity. Pigs in heat stressed treatments were exposed to a cyclical environmental temperature of 12 hours at 35°C±1.2°C with 27% to 45% relative humidity and 12 hours at 30°C±1.1°C with 24% to 35% relative humidity for 21 days.

A feed additive containing >90% sodium saccharide and 0.18% capsaicinoids (CAPS-SUC; TakTik X-Hit, Pancosma, Switzerland) was supplemented by mixing into feed at an inclusion rate of 0.1g/kg for the corresponding treatments upon arrival. Feed intake was recorded daily through a weigh back method by weighing feeders containing refused feed at 8am subtracted by empty feeder weight. Refused and discarded feed was subtracted from the amount given the previous day to determine feed intake. After the start of environmental treatments, the percent reduction in feed intake for pigs in the heat stress environment were determined daily for both dietary treatments (HS-and HS+), based on the animals average feed intake during the thermal neutral acclimation week. The average percent reduction for each treatment was then applied individually to the pair-fed animals accordingly.

Temperature and humidity of each room were measured (Acurite® model Thermal Hygro, Bellingham, WA) and recorded three times daily (8am, 2pm, and 8pm). Heat and constant air circulation were maintained in the heat stress room using an electric forced air heater (model F3F551QT, TPI Corporation). Animals were monitored for signs of distress throughout the experiment three times daily (8am, 2pm, and 8pm). Rectal temperatures were monitored with

(SureTemp ® Plus, Welch Allyn, Skaneateles Falls, NY) and respiration rates (breaths/minute) via visual observation and a stopwatch were recorded at these times. Body weights were recorded upon arrival, before the advent of environmental treatments, and every 3 days after the start of environmental treatments until completion of the study using a Raytec AH300 Way Pig market hog scale (Ephrata, PA).

Skeletal Muscle Biopsy

Skeletal muscle biopsies were performed as described previously (Zhao et al., 2018). Prior to the start of environmental treatments pigs were fasted for 8 hours and placed under general anesthesia using isoflurane. An initial scan with Dual-energy X-ray absorptiometry (DEXA) (Prodigy Advance, General Electric, Boston, MA) was performed while animals were in sternal recumbency. Muscle biopsy of the *Longissimus Dorsi* (LD) was taken at approximately the first lumbar vertebrae. Biopsy sites were shaved and cleaned with betadine and 70% ethanol. A small incision (~1cm) was made with a scalpel and 10 gauge X 9 cm long Varcora Biopsy probe (Bard, Murray Hill, New Jersey) was inserted to collect the biopsy. Muscle samples from the biopsies were used for metabolism analysis at the Metabolic Phenotyping Core at Virginia Tech, and another stored at -80°C for further analysis. Substrate oxidation and metabolic flexibility were performed as previously described (Zhao et al., 2018). After tissue collection, the incisions sites were sutured with PGA Violet Braided Polyglycolic Absorbable Suture (Oasis, Mettawa, IL) and disinfected with Alushield (VetOne, Pasadena, CA).

At the termination of environmental treatments, animals were again DEXA scanned and a muscle biopsy of the contralateral LD was taken. Two days after environmental treatments ceased blood samples were collected, and animals were euthanized with sodium pentabartol under anesthesia. Tissues were collected within 10 minutes of death. The liver and the right

longissimus dorsi (LD) muscle were removed and weighed. A one-inch section of the liver was rinsed with PBS and stored in phosphate buffered formalin for future histological analysis. Sections of the liver and LD were flash frozen in liquid nitrogen and stored at -80°C for further analysis.

Blood Chemistry

Blood samples were collected from the jugular vein using two 10mL BD vacutainer tubes containing either lithium heparin or EDTA (BD, Franklin Lakes NJ) under general anesthesia with isoflurane before the onset of environmental treatment and once every week for the remainder of the study. After each blood sample collection, plasma was separated from blood from the lithium heparin tube spun at 1500 x g at 4°C for 10 minutes aliquoted and stored at -80°C or sent to the Virginia-Maryland Regional College of Veterinary Medicine Animal Laboratory Services for blood metabolite analysis using a small animal profile (SAP), along with whole blood with EDTA for complete blood count (CBC). The SAP measured glucose, urea nitrogen, creatine, phosphorus, calcium, total protein, albumin, globulin, alanine aminotransferase, alkaline phosphatase (ALKP), Gamma-glutamyl transferase (GGT), total bilirubin, creatine kinase, cholesterol, triglycerides, sodium, potassium, chloride, CO2, and anion gap. The CBC measured red blood cell count (RBC), hemoglobin, hematocrit, pack cell volume (PCV), mean corpuscular volume (MVC), mean cell hemoglobin (MVH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW), nucleated red blood cells (nRBC), reticulocyte number, reticulocyte percent, blood cell count, monocytes, basophils, platelets, mean platelet volume (MPV), plasma protein, and fibrogenin.

Statistical Analysis

Data analysis was performed using PRISM analytical software (version 8.1.0 for Windows, GraphPad Software, La Jolla California USA). Sequential measures were analyzed through a mixed-effects model (REML) with time (daily: weight, RR, rectal temperature, and feed intake/weekly: blood chemistry/pre-post: skeletal muscle metabolism) factored as a repeated measure for individual animals. Single point measures were evaluated using one-way ANOVA. Tukey multiple comparison analysis was used to evaluated differences between individual groups within the mixed-effects and one-way ANOVA models. The treatments TN, PF, and HS were compared with 2-way ANOVA with repeated measures when applicable. PF, PF+, HS, HS+ were analyzed with a 3-way ANOVA repeated measure ANOVA or 2-way ANOVA for single point measures. Statistical significance was set at an alpha value of 0.05.

Results

Rectal Temperature and Respiratory Rates

Environmental conditions significantly altered rectal temperature (Figure 2) measures for overall average ($P<0.001$), daily high average ($P<0.001$), and daily low average ($P<0.001$). With HS increasing all measures and PF decreasing all measures compared to TN controls (Table 5.1). Administration of product significantly altered average low temperature ($P=0.032$) driven mainly by increased average low temperatures of supplemented PF pigs (+0.29^{o}C, $P<0.001$). This increase in average low temperature of supplements PF pigs led to a significant interaction between product and environment for average temperatures ($P=0.024$) and average low temperatures ($P=0.005$). RRs were also significantly altered by environment ($P<0.0001$), with RRs of HS pigs nearly double those of TN and PF pigs. Product supplementation presented no detectible influence over RR (Table 5.2).

Growth Performance

Feed intake (P<0.001), feed efficiency (P <0.001), and average daily gain (P <0.001) were significantly altered by environment (Figure 5.3). Feed intake was reduced in HS- pigs by ~12% which was matched in PF- pig (Table 5.1). Supplementation did not affect feed intake (Table 2). Differences in feed efficiency were driven by decreases in PF- pigs (34%) as the feed efficiency of HS- pigs did not statistically differ from TN (50% vs 59%, respectively). These results were mirrored by average daily gain with PF- pigs presenting with a much lower daily growth rates (0.66 kg/day) compared to HS- and TN pigs (0.54 kg/day vs 0.66 kg/day respectively). Supplementation trended to increase feed efficiency (P =0.083) but had no effect on average daily gain. Neither environment nor supplementation influenced body-fat percentage (P =0.674), fat mass (P =0.307), lean mass (P=0.1808), or bone mineral content (P=0.184) (Tables 5.3 and 5.4).

Environment altered liver weight (P =0.001) with both HS- and PF- pigs having reduced liver weights compared to TN (Figure 4). This was matched with altered ALT expression due to environment (P=0.039). With PF- increasing ALT expression over HS- and TN pigs (45.4±1.1, 34.3±2.0, and 39.5.29±1.0, respectively). Supplementation significantly altered liver weight (P=0.040) causing a strong interaction (P=0.037) with environment. This interaction was driven mainly by the restoration of liver weight of PF animal due to supplementation (0.80±0.03 kg vs 1.03±0.07kg, PF- vs PF+). Supplementation trended to decrease ALT levels (P=0.0816), with supplementation reducing the ALT levels of PF animals (45.1±1.0 vs 37.4±0.6, P=0.0127). Other liver function markers, bilirubin, urea nitrogen, and ALKP, were unaffected by environment or supplementation (Table 5.5 and 5.6).

Muscle Dynamics

Muscle oxidative measures were highly influenced by environmental conditions (Figure 5.5). PF- pigs exhibited reduced pyruvate oxidation from week one to week four (P=0.003). Pigs under HS exhibited reduced citrate synthase (CS) (P=0.008), and Cytochrome C (CytoC) (P=0.002) activity and trended on a reduction in metabolic flexibility (P=0.054) from week one to four (Figure 5.6). Supplementation reduced pyruvate oxidation (P=0.012) and exhibited an interaction effect by ameliorating losses in metabolic flexibility of supplement HS pigs (P=0.537).

Blood Chemistry

Blood glucose (P=0.0185), and blood calcium (P=0.049) levels were reduced by HS and PF compared to TN. Creatine kinase (CK) (P=0.002) levels were elevated by HS and reduced by PF compared to TN. An interaction effect was observed between time, environmental condition and, supplementation (P=0.0363) for blood calcium levels driven mainly by the 9.4% increase in supplemented PF animals form week one to week four. An interaction (P=0.026) was also observed between time and supplementation for creatine kinase levels as well as an interaction of time by environment by supplementation (P=0.035).

Discussion

Supplementation with CAP-SUC during acute heat stress improved feed:gain ratio in pigs (Biggs et al., 2020). Due to this, the current study sought to examine the efficacy of CAPS-SUC to mitigate the negative effects heat stress on pig performance and muscle metabolism during chronic heat stress. By design, our heat stress protocol resulted in marked hyperthermia. Our protocol utilized temperatures of 35^{o}C for 12 hours during the day and 30^{o}C for 12 hours during the night to mimic a natural circadian pattern while maintaining a consistently higher heat load than that of the thermal neutral controls. As expected, pigs subjected to heat stress exhibited a 0.4

°C increase in rectal temperature accompanied by a higher respiratory rate than pigs in thermal neutral conditions, which has been consistently observed in both chronic and acute heat stress (Boddicker et al., 2014; Ganesan et al., 2017; Volodina et al., 2017). A persistent, elevated rectal temperature was observed for the duration of the 28-day environmental treatment. Increased respiratory rate in heat stressed animals helps to regulate body temperature during heat stress through evaporative cooling and moisture loss in the airways (Baumgard and Rhoads, 2013b). While CAP-SUC increased respiratory rate in heat stressed animals during acute heat stress (Biggs et al., 2020), no differences between HS- and HS+ were observed in this study. During periods of heat stress, visceral blood flow is directed towards the skin to increase heat loss (Lambert, 2009). Supplementing CAP-SUC may redirect visceral blood flow back to the gut during heat stress by stimulating secretion of GLP-2, which has been shown to increase intestinal blood flow (Guan et al., 2006; Stephens et al., 2006), and may result in the need for increased respiration to dissipate heat instead of through radiant heat dissipation. The diurnal heat stress pattern used in this study was not as aggressive of a heat stress as used in Biggs et al., 2020 which may account for the lack of differences in respiratory rates in supplemented animals. Rectal temperatures in pair-fed animals in thermal neutral condition were lower than control animals. The reduction in body temperature due to pair-feeding may be attributed to the reduced amount of metabolic heat production associated with the decline in plane of nutrition (Pearce et al., 2013b).

In the current study, hyperthermia reduced feed intake regardless of supplementation. A reduction in feed intake due to heat stress has been well documented in pigs, cattle, sheep, chicken, and goats (Yalcin et al., 2001; Rhoads et al., 2009; Sohail et al., 2012; Pearce et al., 2013b; Salama et al., 2014). This immediate reduction in appetite may be an adaptive strategy to minimize metabolic heat production (Collin et al., 2001; Pearce et al., 2013b). Studies have shown nutrient

restriction due to a reduced voluntary feed intake is not the sole cause of the negative effects of heat stress (Pearce et al., 2013b), and caloric restriction alone has its own effects on growth and metabolism. Many of the adverse consequences of heat stress are also observed with a low plane of nutrition, therefore, we used a pair-fed control in thermal neutral conditions to mimic the behavioral reduction in feed intake observed during heat stress to elucidate the indirect effects of plane of nutrition. However, it should be noted that feeding pattern may be different between groups, as heat stressed pigs may eat many small frequent meals throughout the day (Collin et al., 2001; Zhao et al., 2018), while pair-fed pigs were given meals twice daily.

Final body weight was not affected by heat stress with or without supplementation compared to the thermal neutral controls. Average daily gain was reduced in heat stressed and pair-fed animals. Feed efficiency, calculated by the ratio of average weight of gain per day (kg) over average feed consumption per day (kg), was not impacted by heat stress, however, supplementation had a tendency to increase feed efficiency. This difference may indicate that supplementation was able to improve efficiency. This is consistent with our previous study in which supplementation with CAPS-SUC improved feed efficiency in heat stressed and pair-fed pigs (Biggs et al., 2020). Since pair-fed animals were given access to a limited amount of feed, the ability of PF+ animals to be more efficient than PF animals is of importance. Together with PF+ animals exhibiting a higher final body weight suggests that supplementation of CAPS-SUC was able to influence the ability of animals in nutrient restricted conditions to gain weight. It is well documented that reductions in feed intake have deleterious effects on intestinal health (Ferraris and Carey, 2000; Pearce et al., 2013a; Kvidera et al., 2017b), which may be the cause of reduced feed efficiency in PF animals. Supplementing with CAP-SUC has been previously shown to increase in feed efficiency, however, no significant morphological changes to the intestine were

observed, which may be due to a shorter duration of heat stress treatment (Biggs et al., 2020). Previous studies have shown that acute heat stress can reduce the transepithelial electrical resistance, a measure of barrier function and integrity (Pearce et al., 2012). Although the study by Biggs et al. (2020) did not observe differences in intestinal morphology, CAP-SUC may help to improve the intestinal integrity and nutrient transport. Sugars and artificial sweeteners can stimulate the secretion of the gut hormone glucagon-like peptide (GLP)-2 and upregulates expression of SGLT-1 (Drucker, 1996). Glucagon-like peptide-2 is a potent gastroprotective agent that increases blood flow (Stephens et al., 2006), stimulates mucosal growth (Moran et al., 2010; Vegge et al., 2013), reduces the inflammatory response (Cani et al., 2009), prevents cell death (Burrin et al., 2007), and maintains glucose homeostasis (Bahrami et al., 2010; Shi et al., 2013). Supplementing artificial sweeteners upregulates SGLT-1 which enhances gut absorption, and improves gut morphology via increases villus and tight junction strength in pigs and ruminants (Moran et al., 2010; Moran et al., 2014). During feed restriction, GLP-2 which has been shown to improve intestinal morphology (Kvidera et al., 2017b). Due to the voluntary reduction in feed intake that occurs in heat stress and the designed reduction in feed with pair-feeding, supplementing with artificial sweeteners like in CAP-SUC may improve gut function. Furthermore, SGLT-1 is the most sensitive glucose transporter in the gut to oxidative stress (Andrade et al., 2018), which occurs during heat stress. Since artificial sweeteners upregulate SGLT-1, it may provide increased absorption of glucose. Mechanistically, supplementation with CAPS-SUC may improve intestinal morphology and nutrient uptake to improve efficiency in feed restricted animals as seen in the present study and by Biggs et al., 2020.

Maintaining metabolic flexibility is critical for overall metabolic health, loss of which is a key component in heat stress induced conditions (illness, mortality, etc.) (Meex et al., 2010; Zhao

et al., 2018). Feed restricted animals utilize free fatty acids from adipose tissue as an additional energy source as well as a glucose sparing mechanism. In the current study, fatty acid oxidation was not different between treatments pre or post environmental treatments. However, pyruvate oxidation did decrease in treatments except for heat stressed pigs. In our previous study, heat stress decreased metabolic flexibility and limited the ability to use non-esterified fatty acids whereas pair-fed animals maintained metabolic flexibility and increased fatty acid oxidation (Zhao et al., 2018). Consistent with this study, HS- animals did not retain metabolic flexibility. Supplementation supported metabolic flexibility and mitigated the negative effects of environment. Since pair-fed pigs had a similar metabolic flexibility to control pigs, heat stress pigs may not utilize fatty acids as their primary fuel source. This finding is consistent with previous studies that suggest FFAs from adipose tissue do not mobilize during periods of heat stress, and skeletal muscle does not utilize FFA as a fuel source. Activation of toll-like receptor 4 (TLR4) has been shown to alter metabolic flexibility and fatty acid oxidation (Frisard et al., 2010). It is possible that compromised gut integrity due to heat stress shunting blood away from the splenic bed leads to an increase in endotoxin release into the body, therefore causing altered metabolism and through the activation of TLR4.

In the current study, glucose levels in circulation tended to decrease in heat stressed animals compared to control animals. A decrease in circulating glucose is consistent with previous studies using other species including cattle (Nardone et al., 1997; Itoh et al., 1998; Ronchi et al., 1999; O'brien et al., 2010), chickens (Rahimi, 2005), and rats (Mitev et al., 2005). Studies have attributed this decrease in glucose to a heat induced increase in circulating basal insulin concentrations in cattle, pigs and mice (Elsasser et al., 2009; O'brien et al., 2010; Wheelock et al., 2010; Baumgard and Rhoads, 2013b; Biggs et al., 2020). During acute heat stress, CAP-SUC supplementation had

a tendency to decrease circulating glucose levels (Biggs et al., 2020). Previous studies have demonstrated that dietary capsaicin decreases circulating blood glucose, and increases glucose clearance (Karlsson et al., 1994; Tolan et al., 2001; Gram et al., 2005). However, other studies have reported an increase in blood glucose levels after heat stress in chickens (Garriga et al., 2006), rabbits (Marder et al., 1990), and sows (Prunier et al., 1997). It has been demonstrated that during acute heat stress in sows, ileal glucose transport activity increased after 24 hours with a tendency towards and increased GLUT-2 transporters (Pearce et al., 2013c). The response of blood glucose levels during heat stress may differ due to a variety of parameters, including species, duration and magnitude of heat stress, and stage of growth.

Creatinine, creatine kinase (CK), and blood urea nitrogen (BUN) are end products of metabolism and their circulating levels often used as markers of muscle catabolism during stress (Hosten, 1990). In this study, there were no differences in creatinine levels between treatments, however, heat stress increased CK levels. These results are consistent with other heat stress studies (Kachadorian and Johnson, 1972; Marder et al., 1990; Pearce et al., 2013b; Biggs et al., 2020). Previous studies suggest that capsaicin reduced levels of BUN, creatinine, and CK, however in this study, supplementation had no effect on creatinine, CK, or BUN. Capsaicin supplementation reduced the levels of circulating creatinine and CK in mice after exercise demonstrating a decrease in muscle damage (Hsu et al., 2016). In this study BUN did not differ between treatments unlike previous studies in which BUN was initially increased after 1 day of heat stress and was reduced after day 3 (Pearce et al., 2013b). However, it is possible that our sampling timepoint after 7 days of heat stress did not capture this initial spike in BUN.

Alanine amino-transferase (ALT) has been utilized as an indicator of liver necrosis as damaged hepatocytes leak ALT so that activity or amount in blood increase compared to animals

with healthy hepatic function. ALT may also become elevated during muscle damage or necrosis (Janssen et al., 1989). Previous reports have documented increased ALT during heat stress in sheep, goats, cattle, and rabbits (Georgie et al., 1973; More et al., 1980; Kataria and Bhatia, 1991; Abdelatif and Modawi, 1994; Nazifi et al., 2003). Herein, heat stressed animals had lower plasma ALT than control animals, and PF animals had higher ALT than their HS- counterparts. Supplementation further reduced plasma ALT. Gamma-glutamyl transferase (GGT) is an enzyme present in the hepatocytes and bile duct epithelial cells and is also used as an indicator of liver function (Abdelatif and Modawi, 1994). Elevated serum GGT are associated with bile duct lesions and liver damage. In this study, HS- did not increase GGT compared to control animals, however HS+ animals had elevated GGT over their non supplemented counterparts. Interestingly, ectoplasmic GGT has been associated with the generation of reactive oxygen species and oxidative stress (Drozdz et al., 1998), however, the correlation between cellular GGT and circulating GGT is not well understood. A basis for the discrepancy between evident liver health in the current study versus the literature is not readily apparent, however the susceptibility of the liver to damage and/or dysfunction during heat stress warrants further examination.

Heat stress often leads to an immune response, which can reduce growth and productivity (Morrow-Tesch et al., 1994; Hall et al., 2001; Mashaly et al., 2004). Immuno-activation alters the nutrient partitioning away from economically important phenotypes and towards stimulated leukocytes which have a large energetic and amino acid requirement, contributing to reduced profitability (Johnson, 2012; Kvidera et al., 2017a). Capsaicin supplementation in dogs has been shown to reduce monocytes by $2.4x10^{-4}$ compared to control groups (Tolan et al., 2001). However, in a previous study, CAP-SUC supplementation during acute heat stress in pigs resulted in an increase in monocyte count in HS- groups when compared to PF animals (Biggs et al., 2020). This

is consistent with the current study where PF reduced monocyte count compared to other treatments, however, supplementation was able to recover monocyte count in PF+ animals to that of TN animals. Biggs et al., (2020) also reported an increase in basophil levels when animals were supplemented regardless of environmental treatments, however, in the current study, no differences were observed between treatments. A reduction in lymphocyte count has been observed in previous studies when animals were exposed to heat stress treatments in pigs and in chickens (Mashaly et al., 2004; Biggs et al., 2020). The current study did not observe differences in lymphocyte number in heat stress treatments. It is likely that differences in the duration and severity of the heat stress treatments between the studies account for the differential observed immune response. Temporal investigations into the heat stress altered immune response may be critical to therapeutic applications designed to support the immune system in a time-dependent manner during heat stress.

Conclusion

Heat stress is a prevalent problem in agriculture that has marked consequences on animal health and performance. This study tested the efficacy of a capsicum oleoresin and artificial sweetener supplement on its ability to mitigate chronic heat stress and modulate muscle metabolism. Supplementation improved average daily gain during heat stress but did not improve final weight over those supplemented. Interestingly, supplementation supported the maintenance of metabolic flexibility during heat stress, and thus, the ability for differential use of fuel substrates. The benefits of enhanced metabolic flexibility in a production setting and in the face of stressors warrants further investigation.

References

Abdelatif, A., and S. M. Modawi. 1994. Effect of hyperthermia on blood constituents in the domestic rabbit (Lepus cuniculus). Journal of thermal biology 19(6):357-363.

Anderson, J., and D. Bates. 1984. Environmental-management in animal agriculture-Curtis, SE. Amer Veterinary Medical Assoc 1931 N Meacham Rd Suite 100, Schaumburg, IL.

Andrade, N., C. Silva, and F. Martel. 2018. The effect of oxidative stress upon intestinal sugar transport: an in vitro study using human intestinal epithelial (Caco-2) cells. Toxicology Research 7(6):1236-1246. doi: 10.1039/c8tx00183a

Bahrami, J., C. Longuet, L. L. Baggio, K. Li, and D. J. Drucker. 2010. Glucagon-like peptide-2 receptor modulates islet adaptation to metabolic stress in the ob/ob mouse. Gastroenterology 139(3):857-868.

Baumgard, L. H., and R. P. Rhoads. 2013a. Effects of heat stress on postabsorptive metabolism and energetics. Annu. Rev. Anim. Biosci. doi: 10.1146/annurev-animal-031412-103644

Baumgard, L. H., and R. P. Rhoads. 2013b. Effects of heat stress on postabsorptive metabolism and energetics. Annu. Rev. Anim. Biosci. 1(1):311-337.

Biggs, M. E., K. A. Kroscher, L. D. Zhao, Z. Zhang, E. H. Wall, D. M. Bravo, and R. P. Rhoads. 2020. Dietary supplementation of artificial sweetener and capsicum oleoresin as a strategy to mitigate the negative consequences of heat stress on pig performance. Journal of Animal Science doi: 10.1093/jas/skaa131

Bishop-Williams, K. E., O. Berke, D. L. Pearl, K. Hand, and D. F. Kelton. 2015. Heat stress related dairy cow mortality during heat waves and control periods in rural Southern Ontario from 2010–2012. BMC veterinary research 11(1):291.

Boddicker, R. L., J. T. Seibert, J. S. Johnson, S. C. Pearce, J. T. Selsby, N. K. Gabler, M. C. Lucy, T. J. Safranski, R. P. Rhoads, and L. H. Baumgard. 2014. Gestational heat stress alters postnatal offspring body composition indices and metabolic parameters in pigs. PLoS One 9(11):e110859.

Burrin, D. G., B. Stoll, X. Guan, L. Cui, X. Chang, and D. Hadsell. 2007. GLP-2 rapidly activates divergent intracellular signaling pathways involved in intestinal cell survival and proliferation in neonatal piglets. American Journal of Physiology-Endocrinology and Metabolism 292(1):E281-E291. doi: 10.1146/annurev-animal-031412-103644

Cani, P. D., S. Possemiers, T. Van de Wiele, Y. Guiot, A. Everard, O. Rottier, L. Geurts, D. Naslain, A. Neyrinck, and D. M. Lambert. 2009. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut 58(8):1091-1103.

Collin, A., V. J. Milgen, and D.-S. of animal …. 2001. Effect of high temperature and feeding level on energy utilization in piglets. Journal of animal …

D'Allaire, S., R. Drolet, and D. Brodeur. 1996. Sow mortality associated with high ambient temperatures. The Canadian Veterinary Journal 37(4):237.

De Lange, C., J. Pluske, J. Gong, and C. Nyachoti. 2010. Strategic use of feed ingredients and feed additives to stimulate gut health and development in young pigs. Livestock Science 134(1-3):124-134. doi: 10.1016/j.livsci.2010.06.117

Drozdz, R., C. Parmentier, H. Hachad, P. Leroy, G. ́erard Siest, and M. Wellman. 1998. γ-Glutamyltransferase dependent generation of reactive oxygen species from a glutathione/transferrin system. Free Radical Biology and Medicine 25(7):786-792.

Drucker, D. 1996. Ehrlich P, Asa SL, Brubaker PL. Induction of intestinal epithelial proliferation by glucagon-like peptide 2:7911-7916. doi: 10.1073/pnas.93.15.7911

Elsasser, T., R. Rhoads, S. Kahl, R. Collier, L. Baumgard, C. Li, and T. Caperna. 2009. Heat stress augments plasma tyrosine-nitrated proteins and lactate-to-pyruvate ratio after repeated endotoxin (LPS) challenge in steers. J. Anim. Sci 87(9):47-59.

Ferraris, R. P., and H. V. Carey. 2000. Intestinal transport during fasting and malnutrition. Annual review of nutrition 20(1):195-219.

Frisard, M. I., R. P. McMillan, and M.-J. of …. 2010. Toll-like receptor 4 modulates skeletal muscle substrate metabolism. American Journal of …

Ganesan, S., O. Volodina, S. C. Pearce, N. K. Gabler, L. H. Baumgard, R. P. Rhoads, and J. T. Selsby. 2017. Acute heat stress activated inflammatory signaling in porcine oxidative skeletal muscle. Physiological reports 5(16)doi: 10.14814/phy2.13397

Garriga, C., R. R. Hunter, C. Amat, J. M. Planas, M. A. Mitchell, and M. Moretó. 2006. Heat stress increases apical glucose transport in the chicken jejunum. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 290(1):R195-R201.

Georgie, G., D. Chand, and M. Razdan. 1973. Seasonal changes in plasma cholesterol & serum alkaline phosphatase & transaminases activities in cross-bred cattle. Indian journal of experimental biology

Goodpaster, B. H., and L. M. Sparks. 2017. Metabolic flexibility in health and disease. Cell metabolism 25(5):1027-1036. doi: 10.1016/j.cmet.2017.04.015

Gram, D. X., A. J. Hansen, M. Wilken, T. Elm, O. Svendsen, R. D. Carr, B. Ahrén, and C. L. Brand. 2005. Plasma calcitonin gene-related peptide is increased prior to obesity, and sensory nerve desensitization by capsaicin improves oral glucose tolerance in obese Zucker rats. European journal of endocrinology 153(6):963-969. doi: 10.1530/eje.1.02046

Guan, X., H. E. Karpen, J. Stephens, J. T. Bukowski, S. Niu, G. Zhang, B. Stoll, M. J. Finegold, J. J. Holst, and D. L. Hadsell. 2006. GLP-2 receptor localizes to enteric neurons and endocrine cells expressing vasoactive peptides and mediates increased blood flow. Gastroenterology 130(1):150-164. doi: 10.1053/j.gastro.2005.11.005

Hahn, G. 1999. Dynamic responses of cattle to thermal heat loads. Journal of animal science 77(suppl_2):10-20.

Hall, D. M., G. R. Buettner, L. W. Oberley, L. Xu, R. D. Matthes, and C. V. Gisolfi. 2001. Mechanisms of circulatory and intestinal barrier dysfunction during whole body hyperthermia. American Journal of Physiology-Heart and Circulatory Physiology 280(2):H509-H521. doi: 10.1152/ajpheart.2001.280.2.H509

Hansen, P. J. 2009. Effects of heat stress on mammalian reproduction. Philosophical Transactions of the Royal Society B: Biological Sciences 364(1534):3341-3350. doi: doi:10.1098/rstb.2009.0131

Hennessy, D., and P. Williamson. 1984. Stress and summer infertility in pigs. Australian Veterinary Journal 61(7):212-215.

Hosten, A. O. 1990. BUN and creatinine, Clinical Methods: The History, Physical, and Laboratory Examinations. 3rd edition. Butterworths.

Hsu, Y.-J., W.-C. Huang, C.-C. Chiu, Y.-L. Liu, W.-C. Chiu, C.-H. Chiu, Y.-S. Chiu, and C.-C. Huang. 2016. Capsaicin supplementation reduces physical fatigue and improves exercise performance in mice. Nutrients 8(10):648.

Ingram, D. 1965. Evaporative cooling in the pig. Nature 207(4995):415-416.

Itoh, F., Y. Obara, M. T. Rose, H. Fuse, and H. Hashimoto. 1998. Insulin and glucagon secretion in lactating cows during heat exposure. Journal of animal science 76(8):2182-2189.

Iwai, K., A. Yazawa, and T. Watanabe. 2003. Roles as metabolic regulators of the non-nutrients, capsaicin and capsiate, supplemented to diets. Proceedings of the Japan Academy, Series B 79(7):207-212. doi: 10.2183/pjab.79B.207

Janssen, G., H. Kuipers, G. Willems, R. Does, M. Janssen, and P. Geurten. 1989. Plasma activity of muscle enzymes: quantification of skeletal muscle damage and relationship with metabolic variables. International journal of sports medicine 10(S 3):S160-S168.

Johnson, R. W. 2012. Fueling the immune response: what's the cost? In: J. F. Patience, editor, Feed efficiency in swine. Wageningen Academic Publishers, Wageningen. p. 211-223.

Kachadorian, W. A., and R. E. Johnson. 1972. The effects of activity and thermal environment on creatinine clearance. Internationale Zeitschrift für angewandte Physiologie einschließlich Arbeitsphysiologie 30(3):161-170.

Karlsson, S., A. Scheurink, A. B. Steffens, and B. Ahrén. 1994. Involvement of capsaicin-sensitive nerves in regulation of insulin secretion and glucose tolerance in conscious mice. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 267(4):R1071-R1077. doi: 10.1152/ajpregu.1994.267.4.R1071

Kataria, N., and J. Bhatia. 1991. Activity of some enzymes in the serum of dromedary camels. Research in veterinary science 51(2):174-176.

Kim, D. K., H. S. Lillehoj, S. H. Lee, S. I. Jang, and D. Bravo. 2010. High-throughput gene expression analysis of intestinal intraepithelial lymphocytes after oral feeding of carvacrol, cinnamaldehyde, or Capsicum oleoresin. Poultry science 89(1):68-81.

Kvidera, S., M. Dickson, M. Abuajamieh, D. Snider, M. S. Fernandez, J. Johnson, A. Keating, P. Gorden, H. Green, and K. Schoenberg. 2017a. Intentionally induced intestinal barrier dysfunction causes inflammation, affects metabolism, and reduces productivity in lactating Holstein cows. Journal of dairy science 100(5):4113-4127.

Kvidera, S., E. Horst, M. S. Fernandez, M. Abuajamieh, S. Ganesan, P. Gorden, H. Green, K. Schoenberg, W. Trout, and A. Keating. 2017b. Characterizing effects of feed restriction and glucagon-like peptide 2 administration on biomarkers of inflammation and intestinal morphology. Journal of dairy science 100(11):9402-9417.

Lambert, G. P. 2009. Stress-induced gastrointestinal barrier dysfunction and its inflammatory effects. Journal of animal science 87(suppl_14):E101-E108.

Leung, F. W. 2008. Capsaicin-sensitive intestinal mucosal afferent mechanism and body fat distribution. Life sciences 83(1-2):1-5. doi: 10.1016/j.lfs.2008.04.018

Liu, Y., T. Che, M. Song, J. Lee, J. Almeida, D. Bravo, W. Van Alstine, and J. Pettigrew. 2013a. Dietary plant extracts improve immune responses and growth efficiency of pigs experimentally infected with porcine reproductive and respiratory syndrome virus. Journal of animal science 91(12):5668-5679.

Liu, Y., M. Song, T. Che, J. Almeida, J. Lee, D. Bravo, C. W. Maddox, and J. Pettigrew. 2013b. Dietary plant extracts alleviate diarrhea and alter immune responses of weaned pigs experimentally infected with a pathogenic Escherichia coli. Journal of animal science 91(11):5294-5306.

Marai, I., A. El-Darawany, A. Fadiel, and M. Abdel-Hafez. 2007. Physiological traits as affected by heat stress in sheep—a review. Small ruminant research 71(1-3):1-12.

Marder, J., U. Eylath, E. Moskovitz, and R. Sharir. 1990. The effect of heat exposure on blood chemistry of the hyperthermic rabbit. Comparative biochemistry and physiology. A, Comparative physiology 97(2):245-247.

Mashaly, M., G. Hendricks 3rd, M. Kalama, A. Gehad, A. Abbas, and P. H. Patterson. 2004. Effect of heat stress on production parameters and immune responses of commercial laying hens. Poultry science 83(6):889-894. doi: 10.1093/ps/83.6.889

Mayorga, E., J. Ross, A. Keating, R. Rhoads, and L. Baumgard. 2020. Biology of heat stress; the nexus between intestinal hyperpermeability and swine reproduction. Theriogenology doi: 10.1016/j.theriogenology.2020.05.023

McGlone, J. J., W. Stansbury, and L. Tribble. 1988. Management of lactating sows during heat stress: effects of water drip, snout coolers, floor type and a high energy-density diet. Journal of Animal Science 66(4):885-891.

Meex, R. C. R., V. B. Schrauwen-Hinderling, E. Moonen-Kornips, G. Schaart, M. Mensink, E. Phielix, T. van de Weijer, J.-P. Sels, P. Schrauwen, and M. K. C. Hesselink. 2010. Restoration of Muscle Mitochondrial Function and Metabolic Flexibility in Type 2 Diabetes by Exercise Training Is Paralleled by Increased Myocellular Fat Storage and Improved Insulin Sensitivity. Diabetes 59(3):572-579. doi: 10.2337/db09-1322

Mitev, S., S. Dinevska-Kovkarovska, and B. Miova. 2005. Effect of the acclimation to high environmental temperature on the activity of hepatic glycogen phosphorylase (a+ b and a), liver glycogen content and blood glucose level in rats. Journal of Thermal Biology 30(8):563-568.

Moran, A., M. Al-Rammahi, C. Zhang, D. Bravo, S. Calsamiglia, and S. Shirazi-Beechey. 2014. Sweet taste receptor expression in ruminant intestine and its activation by artificial sweeteners to regulate glucose absorption. Journal of dairy science 97(8):4955-4972. doi: 10.3168/jds.2014-8004

Moran, A. W., M. A. Al-Rammahi, D. J. Batchelor, E. A. Coulter, K. Daly, D. Bravo, and S. P. Shirazi- Beeckey. 2010. Expression of Na+/glucose co-transporter 1 (SGLT1) is enhanced by supplementation of the diet of weaning piglets with artificial sweeteners. British Journal of Nutrition doi: 10.1017/S0007114510000917

More, T., A. Rai, and M. Singh. 1980. Note on certain biochemical responses of sheep and goats exposed to thermal stress. Indian Journal of Animal Sciences 50(11):1012-1014.

Morrow-Tesch, J. L., J. J. McGlone, and J. L. Salak-Johnson. 1994. Heat and social stress effects on pig immune measures. Journal of animal science 72(10):2599-2609. doi: 10.2527/1994.72102599x

Nardone, A., N. Lacetera, U. Bernabucci, and B. Ronchi. 1997. Composition of colostrum from dairy heifers exposed to high air temperatures during late pregnancy and the early postpartum period. Journal of dairy Science 80(5):838-844.

Nardone, A., B. Ronchi, N. Lacetera, and U. Bernabucci. 2006. Climatic effects on productive traits in livestock. Veterinary Research Communications 30:75-81.

Nazifi, S., M. Saeb, E. Rowghani, and K. Kaveh. 2003. The influences of thermal stress on serum biochemical parameters of Iranian fat-tailed sheep and their correlation with triiodothyronine (T 3), thyroxine (T 4) and cortisol concentrations. Comparative Clinical Pathology 12(3):135-139.

Nevius, E., P. Srivastava, and S. Basu. 2012. Oral ingestion of capsaicin, the pungent component of chili pepper, enhances a discreet population of macrophages and confers protection from autoimmune diabetes. Mucosal immunology 5(1):76. doi: 10.1038/mi.2011.50

Nienaber, J., G. Hahn, R. Eigenberg, R. Korthals, J. T. Yen, and D. Harris. 1997. Genetic and heat stress interaction effects on finishing swine. Livestock environment V 2

O'brien, M., R. Rhoads, S. Sanders, G. Duff, and L. Baumgard. 2010. Metabolic adaptations to heat stress in growing cattle. Domestic animal endocrinology 38(2):86-94. doi: 10.1016/j.domaniend.2009.08.005

Patience, J. F., J. F. Umboh, and C.-R. K. Production …. 2005. Nutritional and physiological responses of growing pigs exposed to a diurnal pattern of heat stress. Livestock Production …

Pearce, S., V. Mani, R. Boddicker, J. Johnson, T. Weber, J. Ross, L. Baumgard, and N. Gabler. 2012. Heat stress reduces barrier function and alters intestinal metabolism in growing pigs. Journal of Animal Science 90(suppl_4):257-259. doi: 10.2527/jas52339

Pearce, S., V. Mani, T. Weber, R. Rhoads, J. Patience, L. Baumgard, and N. Gabler. 2013a. Heat stress and reduced plane of nutrition decreases intestinal integrity and function in pigs. Journal of Animal Science 91(11):5183-5193. doi: 10.1371/journal.pone.0070215

Pearce, S. C., N. K. Gabler, J. W. Ross, J. Escobar, J. F. Patience, R. P. Rhoads, and L. H. Baumgard. 2013b. The effects of heat stress and plane of nutrition on metabolism in growing pigs. Journal of animal science 91(5):2108-2118. doi: 10.2527/jas.2012-5738

Pearce, S. C., V. Mani, R. L. Boddicker, J. S. Johnson, T. E. Weber, J. W. Ross, R. P. Rhoads, L. H. Baumgard, and N. K. Gabler. 2013c. Heat stress reduces intestinal barrier integrity and favors intestinal glucose transport in growing pigs. PloS one 8(8):e70215.

Prunier, A., M. M. de Braganca, and J. Le Dividich. 1997. Influence of high ambient temperature on performance of reproductive sows. Livestock Production Science 52(2):123-133.

Rahimi, G. 2005. Effect of heat shock at early growth phase on glucose and calcium regulating axis in broiler chickens. Int. J. Poult. Sci 4:790-794.

Renaudeau, D., J. Gourdine, B. Silva, and J. Noblet. 2008. Nutritional routes to attenuate heat stress in pigs. Livestock and Global Climate Change:134.

Renaudeau, D., J. L. Gourdine, and N. R. St-Pierre. 2011. A meta-analysis of the effects of high ambient temperature on growth performance of growing-finishing pigs. Journal of Animal Science 89(7):2220-2230. doi: 10.2527/jas.2010-3329

Rhoads, M., R. Rhoads, M. VanBaale, R. J. Collier, S. Sanders, W. J. Weber, B. A. Crooker, and L. Baumgard. 2009. Effects of heat stress and plane of nutrition on lactating Holstein cows: I. Production, metabolism, and aspects of circulating somatotropin. Journal of dairy science 92(5):1986-1997.

Ronchi, B., N. Lacetera, U. Bernabucci, A. Nardone, and A. Verini Supplizi. 1999. Distinct and common effects of heat stress and restricted feeding on metabolic status of Holstein heifers. Zootecnica e Nutrizione Animale (Italy)

Salama, A., G. Caja, S. Hamzaoui, B. Badaoui, A. Castro-Costa, D. Facanha, M. Guilhermino, and R. Bozzi. 2014. Different levels of response to heat stress in dairy goats. Small Ruminant Research 121(1):73-79.

Shi, X., F. Zhou, X. Li, B. Chang, D. Li, Y. Wang, Q. Tong, Y. Xu, M. Fukuda, and J. J. Zhao. 2013. Central GLP-2 enhances hepatic insulin sensitivity via activating PI3K signaling in POMC neurons. Cell metabolism 18(1):86-98.

Silva, B., R. Oliveira, J. Donzele, H. Fernandes, M. Abreu, J. Noblet, and C. Nunes. 2006. Effect of floor cooling on performance of lactating sows during summer. Livestock Science 105(1-3):176-184.

Sohail, M., M. Hume, J. Byrd, D. Nisbet, A. Ijaz, A. Sohail, M. Shabbir, and H. Rehman. 2012. Effect of supplementation of prebiotic mannan-oligosaccharides and probiotic mixture on growth performance of broilers subjected to chronic heat stress. Poultry science 91(9):2235-2240.

Stephens, J., B. Stoll, J. Cottrell, X. Chang, M. Helmrath, and D. G. Burrin. 2006. Glucagon-like peptide-2 acutely increases proximal small intestinal blood flow in TPN-fed neonatal piglets. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 290(2)doi: 10.1152/ajpregu.00588.2005

Tolan, I., D. Ragoobirsingh, and E. S. A. Morrison. 2001. The effect of capsaicin on blood glucose, plasma insulin levels and insulin binding in dog models. Phytotherapy research 15(5):391-394. doi: 10.1002/ptr.750

Vegge, A., T. Thymann, P. Lund, B. Stoll, S. B. Bering, B. Hartmann, J. Jelsing, N. Qvist, D. G. Burrin, and P. B. Jeppesen. 2013. Glucagon-like peptide-2 induces rapid digestive adaptation following intestinal resection in preterm neonates. American Journal of Physiology-Gastrointestinal and Liver Physiology 305(4):G277-G285. doi: 10.1152/ajpgi.00064.2013

Volodina, O., S. Ganesan, and P.-S. C. Physiological …. 2017. Short-term heat stress alters redox balance in porcine skeletal muscle. Physiological … doi: 10.14814/phy2.13267

Wheelock, J., R. Rhoads, M. VanBaale, S. Sanders, and L. Baumgard. 2010. Effects of heat stress on energetic metabolism in lactating Holstein cows. Journal of dairy science 93(2):644-655.

Wilkins, I. A., and D. W. Wheeler. 2004. Regulation of temperature. Surgery (Oxford) 22(7):168. doi: 10.1383/surg.22.7.168a.38354

Yalcin, S., S. Özkan, L. Türkmut, and S.-P. B. Science. 2001. Responses to heat stress in commercial and local broiler stocks. 1. Performance traits. British Poultry Science doi: 10.1080/00071660120048375

Yan, Y.-E., Y.-Q. Zhao, H. Wang, and M. Fan. 2006. Pathophysiological factors underlying heatstroke. Medical hypotheses 67(3):609-617. doi: 10.1016/j.mehy.2005.12.048

Yonei, Y., P. Holzer, and P. H. Guth. 1990. Laparotomy-induced gastric protection against ethanol injury is mediated by capsaicin-sensitive sensory neurons. Gastroenterology 99(1):3-9.

Zhao, L., R. P. McMillan, G. Xie, S. Giridhar, L. H. Baumgard, S. El-Kadi, J. Selsby, J. Ross, N. Gabler, M. W. Hulver, and R. P. Rhoads. 2018. Heat stress decreases metabolic flexibility in skeletal muscle of growing pigs. Am J Physiol Regul Integr Comp Physiol 315(6):R1096-r1106. doi: 10.1152/ajpregu.00404.2017

Figures

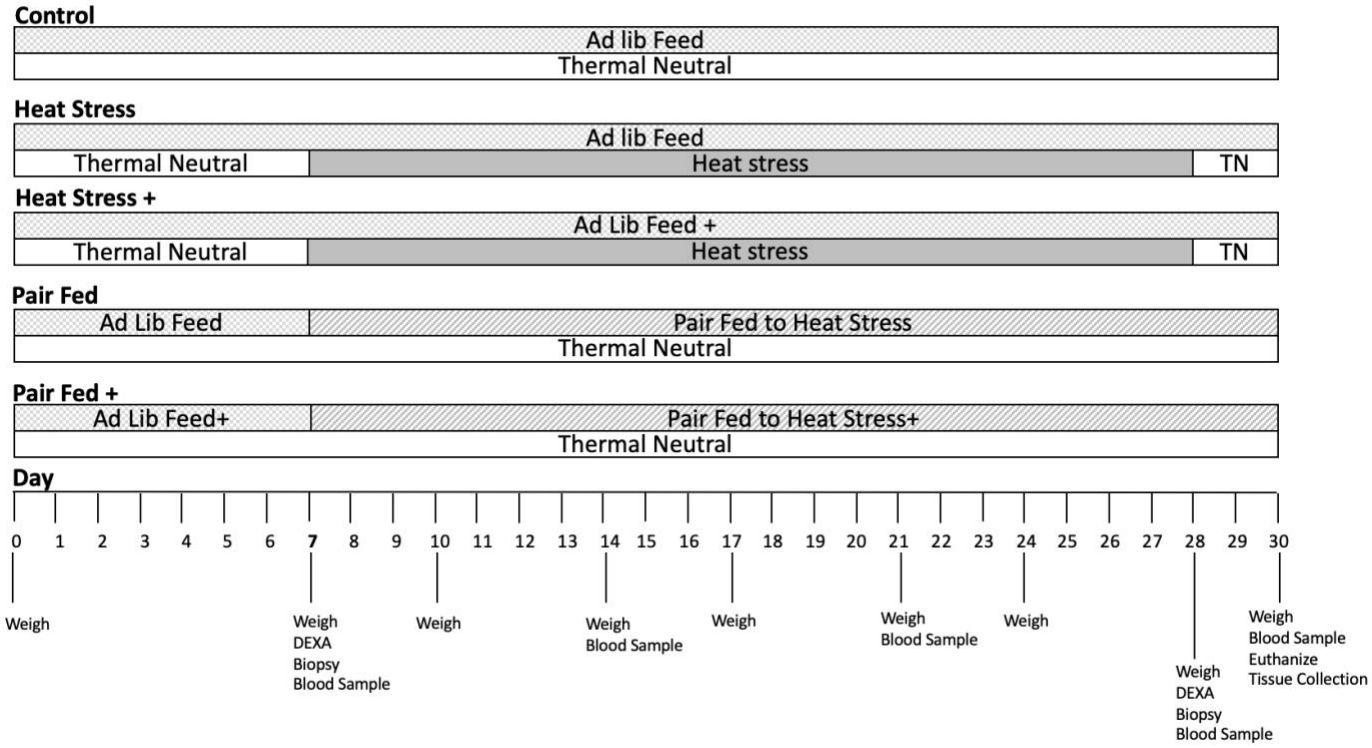

Figure 5.1 Schematic representation of experimental timeline

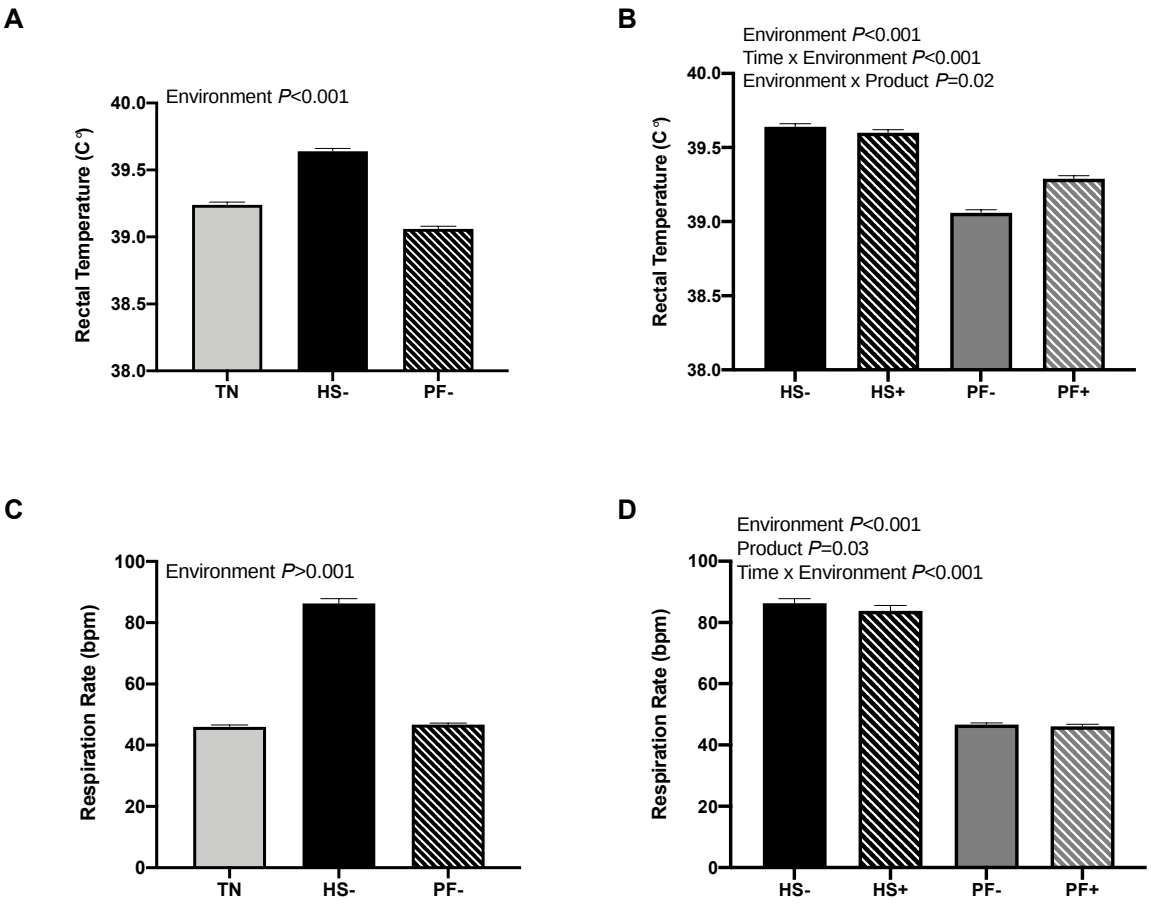

Figure 5.2 The effects of a 21 day heat stress treatment on rectal temperatures and respiratory rates on CAPS-SUC supplemented and non-supplemented pig. Average rectal temperatures (A,B), and respiratory rates (C,D) of pigs subjected to HS- (heat stress), HS+ (heat stress with supplementation), PF (Pair-fed to heat stress), PF+ (Pair-fed to heat stress with supplementation), and TN (thermal neutral, ad. lib. fed). Data are reported as means ± SEM.

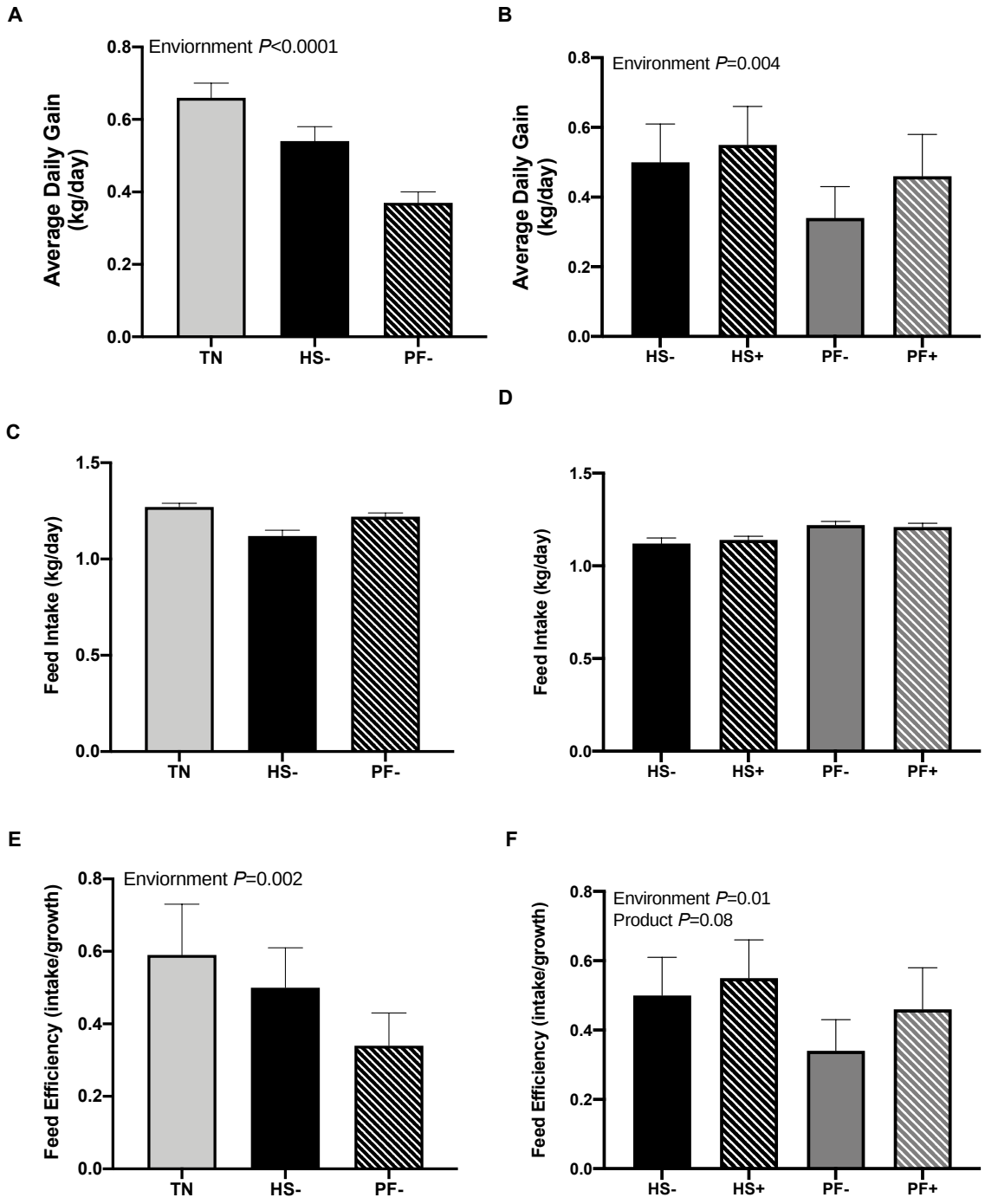

Figure 5.3 Growth performance of pigs after a 21 day heat stress treatment with or without CAPS-SUC supplementation. Average group performance values for average daily gain (A,B), average daily feed intake (C,D), and average feed efficiency (E,F) of pigs assigned to HS- (heat stress), HS+ (heat stress with supplementation), PF (Pair-fed to heat stress), PF+ (Pair-fed to heat stress with supplementation), or TN (thermal neutral, ad. lib. fed). Data are reported as means ± SEM.

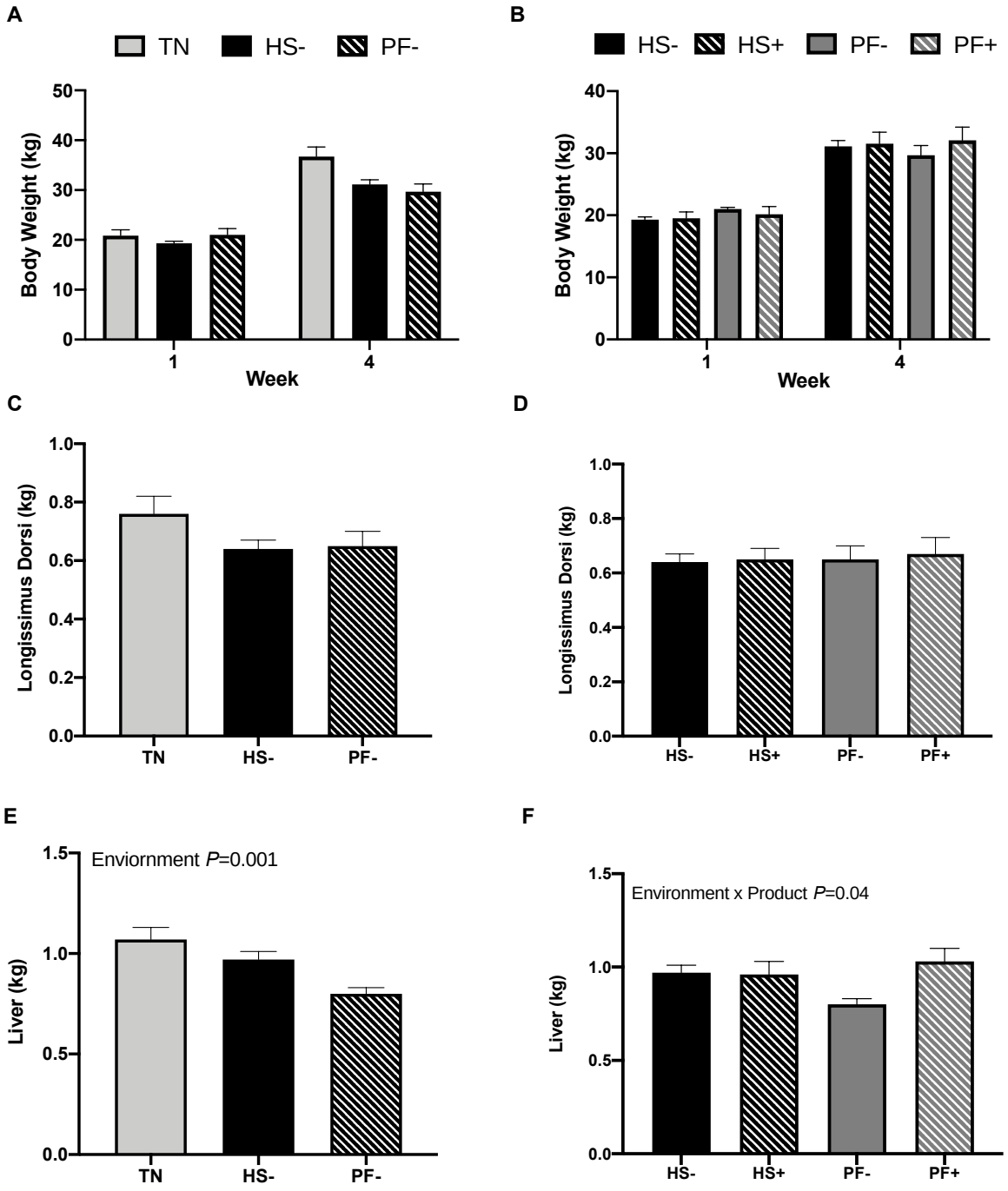

Figure 5.4 The effects heat stress on body, liver, and longissimus dorsi weight of CAPS-SUC supplemented and non-supplemented pigs. Average group performance values weight (A,B), *longissimus dorsi* weight (C,D), liver weight (E,F) of pigs assigned to HS- (heat stress), HS+ (heat stress with supplementation), PF (Pair-fed to heat stress), PF+ (Pair-fed to heat stress with supplementation), or TN (thermal neutral, ad. lib. Fed) Data are reported as means SEM.

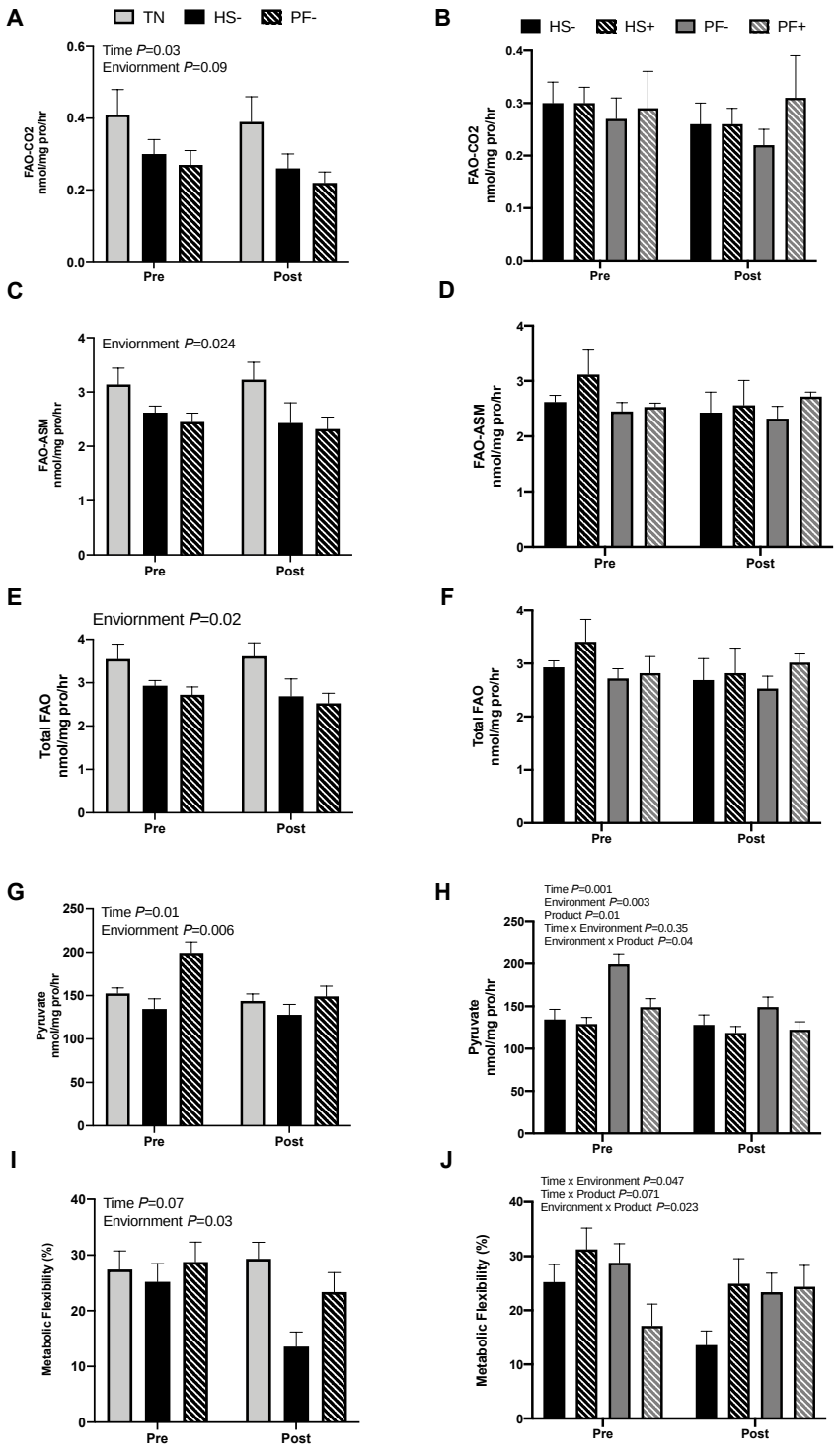

Figure 5.5 The effects of heat stress on fatty acid oxidation on pigs with or without CAPS-SUC supplementation. Pre and post heat stress values of complete fatty acid oxidation (FAO) (A,B), incomplete FAO (C,D), total FAO, (E,F), pyruvate oxidation (G,H), and metabolic flexibility (I,J) of pigs assigned to HS- (heat stress), HS+ (heat stress with supplementation), PF (Pair-fed to heat stress), PF+ (Pair-fed to heat stress with supplementation), or TN (thermal neutral, ad. lib. fed). Data are reported as means ±SEM.

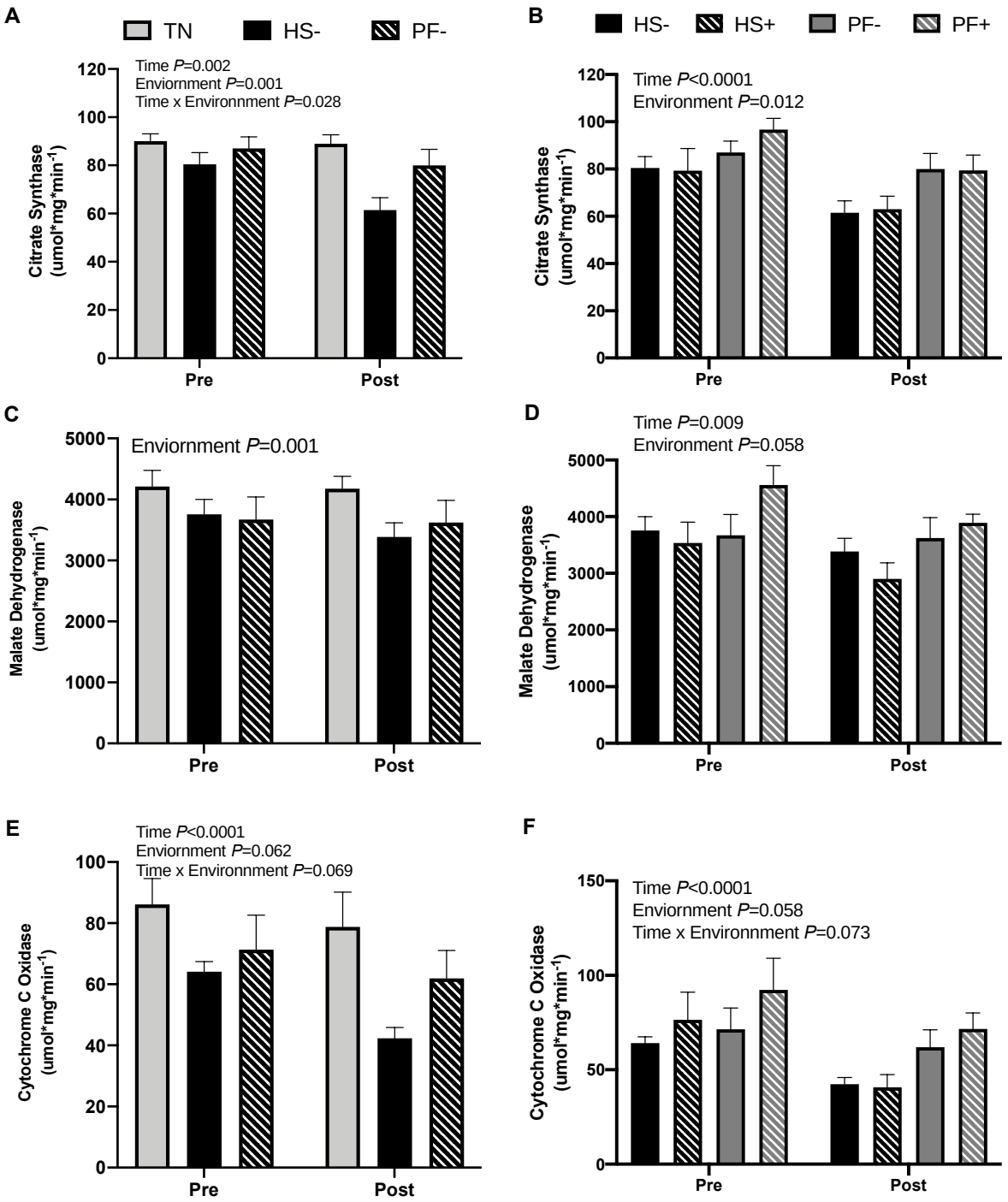

Figure 5.6 The effects of heat stress on enzyme function on pigs with or without CAPS-SUC supplementation. Pre and post heat stress values of enzyme function for citrate synthase (A,B), malate dehydrogenase (C,D), and cytochrome C oxidase (E,F) of pigs assigned to HS- (heat stress), HS+ (heat stress with supplementation), PF (Pair-fed to heat stress), PF+ (Pair-fed to heat stress with supplementation), or TN (thermal neutral, ad. lib. fed). Data are reported as means ±SEM.

Tables

Table 5.1 Effects of heat stress supplementation on growth parameters and body composition measures in pigs.

					P-value		
Measure	Week	Thermal Neutral	Heat Stress	Pair Fed to HS	Time	Environment	Time x Environment
Weight (kg)	1	20.86±1.14	19.31±0.43	21.00±1.28	<0.001****	0.154	<0.001****
	4	36.71±1.92	31.13±0.93	29.69±1.55			
Average Temp (°C)	1-4	39.24±0.02	39.64±0.02	39.06±0.02	0.008**	<0.001****	0.006**
Avg. High Temp (°C)	1-4	39.44±0.03	39.95±0.03	39.32±0.02	0.052"	<0.001****	0.010*
Avg. Low Temp (°C)	1-4	38.98±0.04	39.32±0.02	38.77±0.03	0.158	<0.001****	0.005**
Respiratory rate (BPM)	1-4	46.01±0.54	86.32±1.52	46.72±0.49	0.035*	<0.001****	<0.001****
Feed Intake (kg/day)	1-4	1.27±0.02	1.12±0.03	1.22±0.02	0.057	0.357	<0.001***
Feed efficiency (intake/growth)	1-4	0.59±0.14	0.50±0.11	0.34±0.09	<0.001****	0.002**	0.042*
Avg. Daily Gain (kg/day)	1-4	0.66±0.04	0.54±0.04	0.37±0.03		<0.001***	
LD (kg)	4	0.76±0.06	0.64±0.03	0.65±0.05		0.186	
Liver (kg)	4	1.07±0.06	0.97±0.04	0.80±0.03		0.001**	

Measures of animal performance after 21-day treatment of heat stress, Pair-fed to heat stress, and thermal neutral, ad. lib. fed. Data are reported as means ± SEM. Statistical differences were accepted as significant at $P<0.05$ or a tendency at $P<0.10$.

Table 5.2 Effects of heat stress and CAPS-SUC supplementation on growth parameters and body composition measures in pigs.

						P-value						
Measure	Week	Heat Stress	Heat Stress + Product	Pair Fed to HS	Pair Fed to HS + Product	Time	Environment	Product	Time x Environment	Time x Product	Environment x Product	Time x Environment x Product
Weight (kg)	1	19.31±0.43	19.5±1.06	21±1.28	20.17±1.25	<0.001****	0.869	0.576	<0.001****	0.054"	0.741	0.103
	4	31.13±0.93	31.56±1.81	29.69±1.55	32.08±2.12							
Average Temp (°C)	1-4	39.64±0.02	39.6±0.02	39.06±0.02	39.29±0.02	0.009**	<0.001****	0.113	<0.001****	0.892	0.024*	0.764
Avg. High Temp (°C)	1-4	39.95±0.03	39.92±0.03	39.32±0.02	39.51±0.04	0.041*	<0.001****	0.329	<0.001****	0.35	0.158	0.248
Avg. Low Temp (°C)	1-4	39.32±0.02	39.28±0.02	38.77±0.03	39.06±0.02	0.286	<0.001****	0.032*	0.002**	0.494	0.005**	0.821
Respiratory rate (BPM)	1-4	86.32±1.52	83.83±1.7	46.72±0.49	46.15±0.65	<0.001***	<0.001****	0.697	<0.001****	0.751	0.801	0.768
Feed Intake (kg/day)	1-4	1.12±0.03	1.14±0.02	1.22±0.02	1.21±0.02	0.019*	0.249	0.947	<0.001****	0.771	0.757	0.868
Feed efficiency (intake/growth)	1-4	0.50±0.11	0.55±0.11	0.34±0.09	0.46±0.12	<0.001****	0.013*	0.083"	0.454	0.444	0.57	0.162
Avg. Daily Gain (kg/day)	1-4	0.54±0.04	0.55±0.04	0.37±0.03	0.48±0.04	0.29	0.004**	0.124			0.184	
LD (kg)	4	0.64±0.03	0.65±0.04	0.65±0.05	0.67±0.06		0.713	0.825			0.967	
Liver (kg)	4	0.97±0.04	0.96±0.07	0.8±0.03	1.03±0.07		0.331	0.040*			0.037*	

Measures of animal body composition after 21-day treatment of heat stress, heat stress with supplementation, Pair-fed to heat stress, Pair-fed to heat stress with supplementation. Data are reported as means ± SEM. Statistical differences were accepted as significant at $P<0.05$ or a tendency at $P<0.10$.

Table 5.3 Effects of heat stress on body composition

					P-value		
Measure	Week	Thermal Neutral	Heat Stress	Pair-fed to HS	Time	Environment	Time x Environment
Body Fat%	1	6.13±0.47	5.7±0.27	6.4±0.51	<0.0001****	0.6737	0.0089**
	4	9.57±0.65	8.78±0.41	8.55±0.61			
Tissue (g)	1	21322±1256.12	19984.13±465.46	21470.25±1482.64	<0.0001****	0.1793	<0.0001****
	4	35935.57±1777.9	32032.25±998.8	29287.63±1406.44			
Fat (g)	1	1343.86±170.36	1145.38±65.64	1431.75±203.94	<0.0001****	0.307	0.0013**
	4	3495.86±363.22	2829.5±174.63	2581.88±294.41			
Lean (g)	1	19978.43±1102.25	18838.75±435.75	20042.25±1285.98	<0.0001****	0.1808	<0.0001 ****
	4	32439.86±1486.82	29202.63±903.97	26906.25±1051.12			
Bone Mineral Composition (g)	1	286.01±20.13	262.31±11.1	275.03±20.91	<0.0001****	0.2805	0.0509"
	4	487.41±35.79	415.78±22.07	417.73±24.42			
Fat Free (g)	1	20264.43±1120.51	19226.13±452.28	20317.38±1302.71	<0.0001****	0.1842	<0.0001****
	4	32927.29±1518.32	29618.38±920.2	27324±1072.61			

Measures of animal body composition after 21-day treatment of heat stress, heat stress with supplementation, Pair-fed to heat stress, Pair-fed to heat stress with supplementation. Data are reported as means ± SEM. Statistical differences were accepted as significant at $P<0.05$ or a tendency at $P<0.10$.

Table 5.4 Effects of heat stress and CAP-SUC supplementation on body composition

						P-value						
Measure	Week	Heat Stress	Heat Stress + Product	Pair Fed to HS	Pair Fed to HS + Product	Time	Environment	Product	Time x Environment	Time x Product	Environment x Product	Time x Environment x Product
Body Fat%	1	5.7±0.3	6.18±0.4	6.4±0.5	6.05±0.37	<0.001****	0.921	0.647	0.202	0.389	0.697	0.235
	4	8.78±0.41	9.13±0.56	8.55±0.61	8.97±0.87							
Tissue (g)	1	19984.1±465.5	20245.1±1023.9	21470.3±1482.6	20896.5±1027.9	<0.001****	0.837	0.94	0.422	0.962	0.530	0.162
	4	32032.25±998.8	29682.0±3466.0	29287.6±1406.4	31504.7±1797.3							
Fat (g)	1	1145.4±65.6	1269.38±129.22	1431.8±203.9	1282.0±124.7	<0.001****	0.967	0.576	0.137	0.209	0.895	0.302
	4	2829.5±174.6	3001.3±289.4	2581.9±294.4	2914.8±447.8							
Lean (g)	1	18838.8±435.8	18975.38±907.5	20042.3±1285.9	19614.5±930.9	<0.001****	0.801	0.741	0.003**	0.183	0.783	0.124
	4	29202.63±903.9	29180.88±1419.9	26906.3±1051.1	28589.83±1393.1							
Bone Mineral Composition (g)	1	262.31±11.1	255.05±13.5	275.03±20.9	263.62±17.4	<0.001****	0.648	0.709	0.848	0.814	0.932	0.660
	4	415.78±22.1	405.01±28.4	417.73±24.4	417.82±23.5							
Fat Free (g)	1	19226.1±452.3	19230.4±919.2	20317.4±1302.7	19878.2±947.0	<0.001****	0.788	0.773	0.005**	0.171	0.763	0.157
	4	29618.4±920.2	29586.0±1445.8	27324.0±1072.6	29007.3±1414.6							

Measures of animal performance after 21-day treatment of heat stress, heat stress with supplementation, Pair-fed to heat stress, Pair-fed to heat stress with supplementation. Data are reported as means ± SEM. Statistical differences were accepted as significant at $P<0.05$ or a tendency at $P<0.10$.

Table 5.5 Effects of heat stress on blood chemistry parameter in pigs.

					P-value		
Measure	Week	Thermal Neutral	Heat Stress	Pair Fed to HS	Time	Environ-ment	Time x Environ-ment
Glucose	1	103.57±4.19	104.13±3.68	100±3.19	0.097"	0.019*	0.419
(mg/dL)	2	111.17±2.37	104±6.84	100.88±3.28			
	3	108.17±2.51	98.88±4.59	95.5±2.49			
	4	107.57±3.41	96.88±4	90.5±2.72			
Urea Nitrogen	1	9.86±1.03	12.38±2.46	8.88±0.69	0.490	0.462	0.271
(mg/dL)	2	10±1.1	10.6±0.93	8.75±1			
	3	10.33±1.09	9.5±0.65	9.13±0.61			
	4	9.71±0.89	9±0.63	9.63±0.78			
Creatinine	1	0.69±0.02	0.78±0.13	0.7±0.04	<0.001****	0.205	0.096
(mg/dL)	2	0.73±0.03	0.82±0.01	0.82±0.04			
	3	0.79±0.03	0.95±0.05	0.98±0.05			
	4	0.89±0.04	1.02±0.08	1.09±0.08			
Phosphorous	1	7.43±0.48	7.55±0.32	7.45±0.3	0.795	0.909	0.833
(mg/dL)	2	7.7±0.3	7.24±0.42	7.25±0.25			
	3	7.17±0.32	7.6±0.09	7.56±0.15			
	4	7.37±0.33	7.46±0.32	7.15±0.3			
Calcium	1	10.24±0.25	10.03±0.18	9.85±0.22	0.155	0.049*	0.278
(mg/dL)	2	10.1±0.19	9.98±0.15	9.99±0.26			
	3	10.47±0.2	10.04±0.14	9.84±0.16			
	4	10.87±0.21	10.04±0.17	10.01±0.13			
Total Protein	1	4.83±0.12	4.89±0.2	4.48±0.11	<0.001****	0.526	0.429
(g/dL)	2	5.13±0.13	5.04±0.09	5.06±0.2			
	3	5.47±0.13	5.44±0.11	5.26±0.19			
	4	5.59±0.19	5.45±0.13	5.34±0.22			
Albumin	1	2.41±0.11	2.54±0.11	2.21±0.12	<0.001****	0.364	0.337
(g/dL)	2	2.72±0.14	2.68±0.1	2.56±0.15			
	3	3±0.13	2.94±0.13	2.71±0.14			
	4	3±0.13	2.85±0.13	2.74±0.13			
Globulin	1	2.43±0.12	2.36±0.14	2.26±0.08	0.003**	0.995	0.636
(g/dL)	2	2.42±0.13	2.36±0.04	2.5±0.1			
	3	2.5±0.09	2.51±0.13	2.55±0.1			
	4	2.6±0.15	2.61±0.18	2.63±0.13			
ALT	1	40.29±1.91	37.25±3.48	46.13±4.9	0.029*	0.039*	0.844
(U/L)	2	41.67±3.31	37.6±5.52	46.63±4.27			
	3	39±2.35	33.63±1.65	46.75±5.06			
	4	37.14±1.75	28.88±1.65	42.25±4.05			
ALKP	1	217.14±15.69	224.88±13.71	234.88±10.52	0.005**	0.419	0.398
(U/L)	2	207.17±15.88	187.6±19.21	217.88±8			
	3	188.83±18.89	187.38±20.84	203±13.44			
	4	204.71±19.77	172±20.48	212.38±7.86			
GGT	1	26.57±3	26.38±4.02	29.63±3.34	0.059"	0.331	0.356
(U/L)	2	28.33±2.56	24.2±4.85	40.13±4.32			

	3	29.33±3.53	27.5±4.54	35.25±3.94			
	4	28.71±2.69	26.63±4.52	31.88±3.98			
Total Bilirubin	1	0.09±0.01	0.09±0.01	0.06±0.02	0.185	0.247	0.797
(mg/dL)	2	0.1±0	0.1±0	0.08±0.02			
	3	0.1±0	0.1±0	0.1±0			
	4	0.09±0.01	0.09±0.01	0.09±0.01			
CK	1	614.57±116.37	1223.13±350.33	755.88±130.11	0.148	0.002**	0.111
(U/L)	2	928±285.68	2443.8±724.56	681.75±191.51			
	3	1156.83±416.54	1312.63±283.85	559.13±140.18			
	4	1051.86±227.71	1127.63±233.63	386.38±56.8			
Cholestrol	1	70.71±3.52	70.88±5.36	76.25±2.51	0.174	0.464	0.735
(mg/dL)	2	70±2.29	75.2±6.22	81.88±3.27			
	3	75.17±2.64	73.13±5.81	79.5±3.14			
	4	74.57±3.82	72.38±5.54	77.13±3.84			
TAG	1	49.29±4.52	50.75±5.57	48.75±5.86	0.651	0.536	0.458
(mg/dL)	2	45.5±3.62	48.6±8.02	41.88±2.69			
	3	44.17±5.7	57.13±7.87	44±4.49			
	4	50.14±5.48	44.5±4.53	45.38±2.31			
Sodium	1	141.86±0.34	141.5±1.84	141.25±0.41	0.171	0.023*	0.268
(mEq/L)	2	141.83±0.83	140.8±0.58	141.13±0.58			
	3	141.83±0.54	138.88±0.55	140.63±0.63			
	4	142.14±0.8	138.13±0.67	140.63±0.6			
Potassium	1	3.96±0.09	4.18±0.16	4.06±0.11	0.039*	0.849	<0.001****
(mEq/L)	2	3.8±0.05	4±0.11	3.96±0.07			
	3	3.83±0.07	3.84±0.06	4.11±0.1			
	4	4.39±0.11	3.89±0.06	3.96±0.1			
Chloride	1	102.29±0.52	102.13±0.88	102.75±0.16	0.008**	0.675	0.394
(mEq/L)	2	101.67±0.8	102±0.32	102.13±0.55			
	3	101.83±0.98	101.5±0.53	101.88±0.58			
	4	102±0.58	100.25±0.49	101.13±0.44			
CO2	1	30.71±0.47	29.5±0.63	30.75±0.41	0.178	0.041*	0.172
(mEq/L)	2	30.67±0.56	29.4±0.87	30±0.46			
	3	31.83±0.79	29.13±0.61	30.63±0.42			
	4	29.43±0.53	29±0.8	30.75±0.59			
Anion Gap	1	12.81±0.46	14.05±0.99	11.81±0.21	0.011*	0.176	0.009**
(mEq/L)	2	13.3±0.34	13.4±0.54	12.96±0.35			
	3	12±0.34	12.09±0.62	12.24±0.35			
	4	15.1±0.58	12.75±0.58	12.71±0.62			

Measures blood chemistry during a 21-day treatment of HS- (heat stress), PF- (Pair-fed to heat stress), and TN (thermal neutral, ad. lib. fed). Data are reported as means ± SEM. Statistical differences were accepted as significant at $P<0.05$ or a tendency at $P<0.10$.

Table 5.6 Effects of heat stress and CAPS-SUC supplementation on blood chemistry parameter in pigs.

						P-value						
Measure	Week	Heat Stress	Heat Stress + Product	Pair Fed to HS	Pair Fed to HS + Product	Time	Environ-ment	Product	Time x Environ-ment	Time x Product	Environ-ment x Product	Time x Environ-ment x Product
Glucose	1	104.13±3.68	108.71±5.18	100.00±3.19	96.17±3.1	<0.01***	0.45	0.68	0.13	0.93	0.36	0.18
(mg/dL)	2	104±6.84	99.4±2.66	100.88±3.28	107.5±2.97							
	3	98.88±4.59	98.25±2.14	95.5±2.49	99.67±3.4							
	4	96.88±4	92.13±2.84	90.5±2.72	94.83±1.74							
Urea Nitrogen	1	12.38±2.46	10.29±1.92	8.88±0.69	7.83±1.01	0.37	0.26	0.13	0.07	0.88	0.58	0.77
(mg/dL)	2	10.6±0.93	9.2±1.07	8.75±1	9±0.86							
	3	9.5±0.65	7.88±0.69	9.13±0.61	8.33±0.99							
	4	9±0.63	8.75±0.73	9.63±0.78	8.83±0.91							
Creatinine	1	0.78±0.13	0.78±0.06	0.7±0.04	0.66±0.07	<0.01****	0.32	0.31	0.57	0.86	0.30	0.12
(mg/dL)	2	0.82±0.01	0.9±0.06	0.82±0.04	0.75±0.07							
	3	0.95±0.05	0.97±0.04	0.98±0.05	0.82±0.08							
	4	1.02±0.08	1.06±0.05	1.09±0.08	0.92±0.1							
Phosphorous	1	7.55±0.32	6.46±0.46	7.45±0.3	7.63±0.27	0.14	0.36	0.83	0.34	0.14	0.11	0.37
(mg/dL)	2	7.24±0.42	6.94±0.33	7.25±0.25	7.55±0.36							
	3	7.6±0.09	7.73±0.27	7.56±0.15	7.95±0.28							
	4	7.46±0.32	7.64±0.27	7.15±0.3	7.57±0.37							
Calcium	1	10.03±0.18	10.4±0.38	9.85±0.22	9.38±0.33	0.40	0.27	0.79	0.10	0.53	0.52	0.04*
(mg/dL)	2	9.98±0.15	10.22±0.32	9.99±0.26	9.97±0.23							
	3	10.04±0.14	10.04±0.28	9.84±0.16	9.83±0.21							
	4	10.04±0.17	10.15±0.23	10.01±0.13	10.27±0.23							
Total Protein	1	4.89±0.2	4.87±0.14	4.48±0.11	4.72±0.25	<0.01****	0.43	0.70	0.51	0.44	0.64	0.79
(g/dL)	2	5.04±0.09	5.08±0.16	5.06±0.2	4.97±0.19							
	3	5.44±0.11	5.4±0.17	5.26±0.19	5.4±0.2							
	4	5.45±0.13	5.58±0.14	5.34±0.22	5.55±0.24							
Albumin	1	2.54±0.11	2.44±0.12	2.21±0.12	2.38±0.13	<0.01****	0.54	0.73	0.54	0.13	0.39	0.53
(g/dL)	2	2.68±0.1	2.68±0.19	2.56±0.15	2.55±0.16							
	3	2.94±0.13	2.68±0.18	2.71±0.14	2.7±0.19							

	4	2.85±0.13	2.69±0.18	2.74±0.13	2.78±0.2							
Globulin	1	2.36±0.14	2.39±0.14	2.26±0.08	2.37±0.13	<0.01****	0.89	0.29	0.95	0.19	0.92	0.83
(g/dL)	2	2.36±0.04	2.42±0.07	2.5±0.1	2.45±0.03							
	3	2.51±0.13	2.74±0.16	2.55±0.1	2.7±0.12							
	4	2.61±0.18	2.91±0.22	2.63±0.13	2.8±0.23							
ALT	1	37.25±3.48	29.86±1.94	46.13±4.9	38±3.88	0.077"	<0.01**	0.08"	0.75	0.36	0.37	0.57
(U/L)	2	37.6±5.52	34.2±1.85	46.63±4.27	38.33±3.19							
	3	33.63±1.65	32.63±1.75	46.75±5.06	35.67±4.42							
	4	28.88±1.65	29.63±1.05	42.25±4.05	37.5±5.13							
ALKP	1	224.88±13.71	256.57±20.82	234.88±10.52	243±18.01	<0.01****	0.39	0.82	0.28	0.44	0.23	0.96
(U/L)	2	187.6±19.21	199.2±13.59	217.88±8	203.83±16.99							
	3	187.38±20.84	196.63±11.72	203±13.44	178.67±16.15							
	4	172±20.48	182.88±11.98	212.38±7.86	196.17±15.21							
GGT	1	26.38±4.02	34.14±2.69	29.63±3.34	34.33±7.5	0.31	0.57	0.56	0.61	0.11	0.09"	0.40
(U/L)	2	24.2±4.85	32.2±3.44	40.13±4.32	28.83±2.54							
	3	27.5±4.54	36.38±2.74	35.25±3.94	33.17±3.84							
	4	26.63±4.52	33.5±2.24	31.88±3.98	27.33±1.82							
Total Bilirubin	1	0.09±0.01	0.09±0.01	0.06±0.02	0.07±0.02	0.02*	0.13	0.99	0.42	0.54	0.75	0.99
(mg/dL)	2	0.1±0	0.08±0.02	0.08±0.02	0.07±0.02							
	3	0.1±0	0.1±0	0.1±0	0.1±0							
	4	0.09±0.01	0.1±0	0.09±0.01	0.1±0							
CK	1	1223±350	571±76	756±130	509±53	0.16	<0.01**	0.70	0.12	0.03*	0.96	0.04*
(U/L)	2	2444±724	1493±456	682±192	569±72							
	3	1313±284	2437±1035	559±140	432±65							
	4	1128±233	1418±429	386±57	388±51							
Cholestrol	1	70.88±5.36	68±4.12	76.25±2.51	68.67±2.75	0.06"	0.23	0.40	0.42	0.32	0.69	0.80
(mg/dL)	2	75.2±6.22	68±3.33	81.88±3.27	76.5±1.23							
	3	73.13±5.81	72±3.34	79.5±3.14	74.5±4.89							
	4	72.38±5.54	73.75±2.66	77.13±3.84	76.17±5.15							
TAG	1	50.75±5.57	54.71±9.76	48.75±5.86	40±6.03	0.88	0.51	0.66	0.33	0.49	0.52	0.10
(mg/dL)	2	48.6±8.02	47.8±10.16	41.88±2.69	58.33±9.15							
	3	57.13±7.87	50.25±4.73	44±4.49	49.33±7.64							
	4	44.5±4.53	48.5±3.81	45.38±2.31	52±5.83							

Sodium	1	141.5±1.84	141.86±0.91	141.25±0.41	141±0.52	<0.01***	0.09"	0.42	0.16	0.88	0.65	0.52
(mEq/L)	2	140.8±0.58	141.4±0.51	141.13±0.58	142.67±0.33							
	3	138.88±0.55	139.25±0.98	140.63±0.63	141±0.77							
	4	138.13±0.67	139.38±0.56	140.63±0.6	139.67±0.71							
Potassium	1	4.18±0.16	4.09±0.17	4.06±0.11	4.33±0.46	0.04*	0.51	0.85	0.91	0.25	0.92	0.29
(mEq/L)	2	4±0.11	3.84±0.07	3.96±0.07	3.88±0.05							
	3	3.84±0.06	3.81±0.05	4.11±0.1	3.8±0.04							
	4	3.89±0.06	4.08±0.16	3.96±0.1	4.05±0.1							
Chloride	1	102.13±0.88	101.57±0.53	102.75±0.16	102.5±0.56	<0.01****	0.42	0.67	0.59	0.87	0.67	0.17
(mEq/L)	2	102±0.32	101.8±0.49	102.13±0.55	102.67±0.49							
	3	101.5±0.53	101.5±0.42	101.88±0.58	101.5±0.43							
	4	100.25±0.49	100.88±0.4	101.13±0.44	99.83±0.91							
CO2	1	29.5±0.63	31.57±0.48	30.75±0.41	30.33±0.42	0.30	0.03*	0.14	0.07"	0.82	0.33	0.19
(mEq/L)	2	29.4±0.87	30±0.45	30±0.46	30.83±0.31							
	3	29.13±0.61	29.75±0.67	30.63±0.42	30.67±0.49							
	4	29±0.8	29.5±0.38	30.75±0.59	31.17±0.7							
Anion Gap	1	14.05±0.99	12.8±0.2	11.81±0.21	12.5±0.64	0.04*	0.29	0.88	0.08"	0.91	0.34	0.44
(mEq/L)	2	13.4±0.54	13.44±0.52	12.96±0.35	13.05±0.56							
	3	12.09±0.62	11.81±0.29	12.24±0.35	12.63±0.87							
	4	12.75±0.58	13.08±0.38	12.71±0.62	12.72±0.4							

Blood chemistry measures during a 21-day treatment of HS- (heat stress), HS+ (heat stress with supplementation), PF (Pair-fed to heat stress), PF+ (Pair-fed to heat stress with supplementation). Data are reported as means ± SEM. Statistical differences were accepted as significant at $P<0.05$ or a tendency at $P<0.10$.

Table 5.7 Effects of heat stress and CAPS-SUC supplementation on complete blood count in pigs.

					P-value		
Measure	Week	Thermal Neutral	Heat Stress	Pair Fed to HS	Time	Environ-ment	Time x Environ-ment
RBC	1	5.66±0.11	5.65±0.28	5.46±0.15	<0.001****	0.479	**0.024***
x10^6 cells/uL	2	5.91±0.17	5.88±0.13	6.4±0.19			
	3	6.06±0.05	5.78±0.13	6.41±0.2			
	4	6.11±0.14	5.8±0.16	6.11±0.22			
Hemoglobin	1	9.59±0.23	9.51±0.38	9.01±0.19	<0.001****	0.559	**0.042***
g/dL	2	10.02±0.25	9.83±0.06	10.29±0.18			
	3	10.41±0.24	10.08±0.22	10.31±0.19			
	4	10.53±0.4	9.9±0.34	9.71±0.28			
Hematocrit	1	31.77±0.73	30.89±1.41	29.34±0.57	0.001**	0.541	**0.039***
%	2	32±0.89	31.6±0.32	33.41±0.61			
	3	33.36±0.78	32.73±0.76	33.13±0.65			
	4	34.09±1.35	31.9±1	31.36±0.89			
PCV	1	30.39±0.7	30.1±1.02	29.19±0.54	<0.001***	0.477	**0.047***
%	2	31.68±0.55	30.81±0.35	33.35±0.86			
	3	33.09±0.74	31.68±0.83	32.6±0.81			
	4	32.47±1.19	30.73±0.92	29.9±0.9			
MCV	1	56.11±1.06	54.8±1.07	53.94±1.22	0.0048**	0.1414	**0.010***
fL	2	54.2±1.67	53.88±1.23	52.39±1.03			
	3	55±1.08	56.65±0.83	51.91±0.86			
	4	55.71±1.28	55.01±1.07	51.45±0.92			
MCH	1	16.93±0.34	16.91±0.35	16.55±0.38	0.505	0.18	0.056"
pg	2	16.98±0.58	16.76±0.4	16.14±0.35			
	3	17.17±0.34	17.45±0.33	16.14±0.31			
	4	17.19±0.38	17.06±0.44	15.93±0.29			
MCHC	1	30.17±0.37	30.86±0.21	30.71±0.16	0.023*	0.964	0.072"
g/dL	2	31.26±0.3	31.08±0.18	30.76±0.13			
	3	31.21±0.14	30.8±0.14	31.14±0.19			
	4	30.89±0.07	31±0.35	30.93±0.14			
RDW	1	N/A	16.9±0.47	14.43±4.2	<0.001****	0.913	0.749
%	2	17.6±0.54	17.26±0.33	17.13±0.36			
	3	17.1±0.46	16.67±0.24	16.94±0.36			
	4	16.47±0.32	16.45±0.22	16.46±0.36			
nRBCs	1	0.71±0.36	1±0.33	0.5±0.33	0.161	0.236	0.249
	2	0.4±0.24	0.38±0.18	0.25±0.16			
	3	1.14±0.34	0.17±0.17	0.25±0.16			
	4	0.43±0.2	0.14±0.14	0.38±0.26			
Reticulocyte #	1	118.17±9.11	135.86±23.91	183.89±33.45	0.011*	0.471	**0.024***
x10^3 cells/uL	2	202.96±33.66	141.53±14.43	135.75±18.47			
	3	163.07±24.59	122.37±23.24	133.61±18.74			
	4	124.16±14.97	106±13.78	77.49±13.64			
Reticulocyte %	1	2.11±0.13	2.98±0.42	3.37±0.6	0.002**	0.757	**0.001****
	2	3.4±0.48	2.39±0.22	2.13±0.3			

	3	2.64±0.4	2.08±0.37	2.07±0.27			
	4	2.02±0.22	1.86±0.27	1.64±0.22			
WBC	1	18.84±1.39	16.87±1.76	15.11±0.95	0.001***	0.129	0.809
x10^3 cells/uL	2	16.56±1.81	12.27±1.2	12.88±0.71			
	3	14.94±1.64	12.14±1.01	12.92±0.6			
	4	14.72±1.24	13.2±1.4	13.28±0.96			
Seg Neutrophil	1	7.15±0.55	8.91±1.72	7.53±0.86	0.016*	0.9445	0.188
x10^3 cells/uL	2	4.68±1.24	4.33±1.11	6.57±0.67			
	3	7.36±1.12	5.97±0.94	4.8±0.52			
	4	6.36±1.37	5.99±1.41	5.52±0.51			
Band Neutrophil	1	0.07±0.04	0.1±0.05	0.04±0.02	0.099"	0.401	0.434
x10^3 cells/uL	2	0±0	0.01±0.01	0±0			
	3	0.1±0.05	0.01±0.01	0.02±0.02			
	4	0.03±0.03	0±0	0.02±0.02			
Lymphocyte	1	10.22±0.86	6.43±0.63	6.6±0.33	0.038*	**0.005****	**0.001****
x10^3 cells/uL	2	10.82±1.65	5.49±1.18	5.36±0.64			
	3	6.11±0.65	4.8±0.49	6.9±0.28			
	4	7.25±0.86	6.05±0.8	6.61±0.77			
Monocytes	1	1.16±0.23	1.02±0.14	0.63±0.13	0.67	0.034*	0.32
x10^3 cells/uL	2	1.78±1.07	0.72±0.15	0.65±0.13			
	3	1.04±0.15	1.19±0.12	0.69±0.15			
	4	0.88±0.19	0.98±0.13	0.66±0.09			
Eosinophils	1	0.18±0.09	0.32±0.14	0.24±0.06	0.30	0.0510"	0.32
x10^3 cells/uL	2	0.14±0.08	0.08±0.04	0.23±0.06			
	3	0.23±0.07	0.13±0.05	0.4±0.09			
	4	0.12±0.05	0.14±0.03	0.34±0.07			
Basophiles	1	0.07±0.04	0.08±0.07	0.06±0.04	0.24	0.25	0.21
x10^3 cells/uL	2	0.31±0.19	0.06±0.03	0.08±0.03			
	3	0.11±0.03	0.03±0.03	0.12±0.05			
	4	0.07±0.05	0.03±0.03	0.09±0.05			
Platelets	1	366.71±44.21	405.38±26.81	454.75±34.58	0.33	0.075"	0.025*
x10^3 cells/uL	2	349.83±101.43	527.75±43.94	460.13±64.25			
	3	398.57±31.43	317.17±41.21	514.25±23.81			
	4	322.86±43.66	400.71±19.01	471.88±52.03			
MPV	1	0±0	3.59±1.76	4.46±1.69	<0.001****	0.045*	0.084"
x10^3 cells/uL	2	7.76±1.95	9.31±0.26	9.96±0.41			
	3	9.03±0.28	10.38±0.3	9.24±0.25			
	4	10.34±0.46	9.39±0.18	9.6±0.29			
Plasma Protein	1	5.2±0.11	5.28±0.23	4.86±0.12	<0.001****	0.27	0.25
g/dL	2	5.78±0.26	5.49±0.1	5.38±0.11			
	3	5.71±0.14	5.85±0.1	5.63±0.19			
	4	5.87±0.18	5.71±0.15	5.49±0.19			
Fibrinogen	1	214.29±40.41	237.5±18.3	200±26.73	0.34	0.56	0.49
mg/dL	2	192±69.74	187.5±22.66	200±26.73			
	3	228.57±18.44	200±44.72	300±37.8			
	4	214.29±26.08	214.29±34.01	237.5±26.31			

Blood chemistry measures during a 21-day treatment of heat stress), pair-fed to heat stress, and thermal neutral, ad. lib. fed. Data are reported as means ± SEM. Statistical differences were accepted as significant at $P<0.05$ or a tendency at $P<0.10$.

Table 5.8 Effects of heat stress and CAPS-SUC supplementation on complete blood count in pigs.

						P-value						
Measure	Week	Heat Stress	Heat Stress + Product	Pair Fed to HS	Pair Fed to HS + Product	Time	Environ-ment	Product	Time x Environ-ment	Time x Product	Environ-ment x Product	Time x Environ-ment x Product
RBC	1	5.65±0.28	5.63±0.23	5.46±0.15	5.66±0.19	<0.001***	0.181	0.552	0.029*	0.217	0.866	0.506
x10^6 cells/uL	2	5.88±0.13	5.71±0.21	6.4±0.19	6.06±0.14							
	3	5.78±0.13	5.95±0.16	6.41±0.2	6.16±0.19							
	4	5.8±0.16	5.83±0.11	6.11±0.22	6.08±0.2							
Hemoglobin	1	9.51±0.38	9.24±0.32	9.01±0.19	9.47±0.18	<0.001***	0.259	0.469	0.027*	0.262	0.200	0.527
g/dL	2	9.83±0.06	9.29±0.26	10.29±0.18	10.15±0.23							
	3	10.08±0.22	9.67±0.29	10.31±0.19	10.2±0.38							
	4	9.9±0.34	9.45±0.17	9.71±0.28	10.12±0.43							
Hematocrit	1	30.89±1.41	30.01±1.06	29.34±0.57	30.4±0.64	<0.001***	0.274	0.525	0.027*	0.543	0.218	0.819
%	2	31.6±0.32	30.01±0.77	33.41±0.61	32.92±0.76							
	3	32.73±0.76	31.47±1.1	33.13±0.65	33.28±1.13							
	4	31.9±1	30.84±0.51	31.36±0.89	32.67±1.31							
PCV	1	30.1±1.02	29.24±0.88	29.19±0.54	30.42±0.75	<0.001***	0.088"	0.529	0.084"	0.387	0.223	0.308
%	2	30.81±0.35	29.5±0.76	33.35±0.86	31.83±1.02							
	3	31.68±0.83	30.4±0.81	32.6±0.81	33.32±1.4							
	4	30.73±0.92	29.34±0.72	29.9±0.9	31.42±1.24							
MCV	1	54.8±1.07	53.59±1.79	53.94±1.22	53.78±0.91	0.091"	0.489	0.959	0.112	0.264	0.232	0.104
fL	2	53.88±1.23	52.89±1.69	52.39±1.03	54.35±0.74							
	3	56.65±0.83	53.06±1.86	51.91±0.86	54±0.76							
	4	55.01±1.07	53.09±1.54	51.45±0.92	53.68±0.72							
MCH	1	16.91±0.35	16.51±0.5	16.55±0.38	16.73±0.31	0.157	0.551	1.000	0.587	0.664	0.202	0.332
pg	2	16.76±0.4	16.36±0.53	16.14±0.35	16.77±0.2							
	3	17.45±0.33	16.31±0.56	16.14±0.31	16.56±0.21							
	4	17.06±0.44	16.28±0.48	15.93±0.29	16.65±0.25							
MCHC	1	30.86±0.21	30.86±0.18	30.71±0.16	30.92±0.33	0.891	0.999	0.547	0.530	0.566	0.767	0.300
g/dL	2	31.08±0.18	30.94±0.16	30.76±0.13	30.85±0.16							
	3	30.8±0.14	30.77±0.33	31.14±0.19	30.6±0.17							

	4	31±0.35	30.65±0.12	30.93±0.14	31±0.16							
RDW	1	16.9±0.47	17.7±0.49	14.43±4.2	22.37±5.04	0.257	0.803	**0.070"**	0.857	**0.021***	0.211	**0.040***
%	2	17.26±0.33	17.6±0.6	17.13±0.36	17.2±0.72							
	3	16.67±0.24	16.96±0.53	16.94±0.36	16.64±0.49							
	4	16.45±0.22	16.75±0.47	16.46±0.36	16.47±0.41							
nRBCs	1	1±0.33	0.29±0.18	0.5±0.33	0.83±0.54	0.057"	0.805	0.609	0.490	0.909	0.392	0.236
	2	0.38±0.18	0.43±0.2	0.25±0.16	0±0							
	3	0.17±0.17	0.14±0.14	0.25±0.16	0.4±0.4							
	4	0.14±0.14	0.13±0.13	0.38±0.26	0.33±0.21							
Reticulocyte #	1	135.86±23.91	141.26±25.44	183.89±33.45	125.88±13.21	<0.001***	0.588	0.639	0.274	0.413	0.292	0.322
x10^3 cells/uL	2	141.53±14.43	173.14±29.52	135.75±18.47	116.4±25.66							
	3	122.37±23.24	126.66±19.1	133.61±18.74	101.98±15.34							
	4	106±13.78	100.2±11.5	77.49±13.64	98.03±11.3							
Reticulocyte %	1	2.98±0.42	2.49±0.41	3.37±0.6	2.21±0.19	<0.001***	0.523	0.555	0.458	**0.009****	0.436	0.808
	2	2.39±0.22	3.05±0.53	2.13±0.3	2.26±0.2							
	3	2.08±0.37	2.14±0.31	2.07±0.27	1.65±0.24							
	4	1.86±0.27	1.71±0.19	1.64±0.22	1.61±0.17							
WBC	1	16.87±1.76	17.33±1.03	15.11±0.95	17.63±1.41	<0.001***	0.779	0.121	0.533	0.348	0.499	0.809
x10^3 cells/uL	2	12.27±1.2	14.15±0.66	12.88±0.71	15.28±1.77							
	3	12.14±1.01	14.11±0.91	12.92±0.6	16.08±2.3							
	4	13.2±1.4	13.44±0.56	13.28±0.96	13.22±1.08							
Seg Neutrophil	1	8.91±1.72	8.55±1.18	7.53±0.86	8.04±1.88	0.004**	0.957	0.482	0.233	0.632	0.711	0.728
x10^3 cells/uL	2	4.33±1.11	5.9±0.87	6.57±0.67	7.06±1.22							
	3	5.97±0.94	6.44±0.99	4.8±0.52	7.05±2.39							
	4	5.99±1.41	5.74±0.34	5.52±0.51	5.32±0.89							
Band Neutrophil	1	0.1±0.05	0.06±0.04	0.04±0.02	0.02±0.02	0.142	0.780	0.594	0.408	**0.083"**	0.584	0.961
x10^3 cells/uL	2	0.01±0.01	0.07±0.05	0±0	0.1±0.1							
	3	0.01±0.01	0±0	0.02±0.02	0±0							
	4	0±0	0±0	0.02±0.02	0.03±0.03							
Lymphocyte	1	6.43±0.63	7.43±0.87	6.6±0.33	8.47±1.1	0.069"	0.459	0.167	0.253	0.454	0.916	0.759
x10^3 cells/uL	2	5.49±1.18	6.93±0.87	5.36±0.64	6.39±1.54							

	3	4.8±0.49	6.39±0.94	6.9±0.28	7.81±1.08							
	4	6.05±0.8	6.6±0.57	6.61±0.77	6.25±0.4							
Monocytes	1	1.02±0.14	0.94±0.25	0.63±0.13	0.81±0.07	0.802	0.397	0.178	0.015*	0.067	**0.022***	0.651
x10^3 cells/uL	2	0.72±0.15	0.84±0.17	0.65±0.13	1.47±0.24							
	3	1.19±0.12	1.02±0.19	0.69±0.15	0.87±0.08							
	4	0.98±0.13	0.76±0.15	0.66±0.09	1.03±0.21							
Eosinophils	1	0.32±0.14	0.3±0.09	0.24±0.06	0.24±0.05	0.365	0.189	0.587	0.086"	0.330	0.199	0.421
x10^3 cells/uL	2	0.08±0.04	0.32±0.12	0.23±0.06	0.17±0.08							
	3	0.13±0.05	0.19±0.09	0.4±0.09	0.21±0.13							
	4	0.14±0.03	0.25±0.05	0.34±0.07	0.45±0.14							
Basophiles	1	0.08±0.07	0.04±0.04	0.06±0.04	0.08±0.03	0.562	0.344	0.426	0.778	0.553	0.990	0.849
x10^3 cells/uL	2	0.06±0.03	0.08±0.04	0.08±0.03	0.08±0.06							
	3	0.03±0.03	0.08±0.03	0.12±0.05	0.14±0.1							
	4	0.03±0.03	0.12±0.04	0.09±0.05	0.15±0.07							
Platelets	1	405.38±26.81	439.71±51.73	454.75±34.58	433.67±42.53	0.139	0.882	0.661	0.005**	0.878	**0.049***	0.345
x10^3 cells/uL	2	527.75±43.94	530.86±28.31	460.13±64.25	390.67±45.4							
	3	317.17±41.21	426.71±60.98	514.25±23.81	415.6±46.6							
	4	400.71±19.01	481.63±44.32	471.88±52.03	350.67±58.67							
MPV	1	3.59±1.76	4.01±1.89	4.46±1.69	2.93±1.86	<0.001***	0.759	0.784	0.525	0.970	0.541	0.875
x10^3 cells/uL	2	9.31±0.26	9.3±0.11	9.96±0.41	9.97±0.38							
	3	10.38±0.3	10.57±0.71	9.24±0.25	8.96±0.47							
	4	9.39±0.18	9.44±0.21	9.6±0.29	9.62±0.25							
Plasma Protein	1	5.28±0.23	5.23±0.14	4.86±0.12	5.1±0.23	<0.001***	0.168	0.780	0.459	0.786	0.504	0.884
g/dL	2	5.49±0.1	5.51±0.13	5.38±0.11	5.48±0.22							
	3	5.85±0.1	5.83±0.14	5.63±0.19	5.78±0.2							
	4	5.71±0.15	5.76±0.13	5.49±0.19	5.62±0.25							
Fibrinogen	1	237.5±18.3	185.71±34.01	200±26.73	200±25.82	0.090"	0.294	0.953	0.086"	0.531	0.579	0.605
mg/dL	2	187.5±22.66	242.86±36.89	200±26.73	200±25.82							
	3	200±44.72	228.57±35.95	300±37.8	300±31.62							

	4	214.29±34.01	212.5±22.66	237.5±26.31	200±25.82

Blood chemistry measures during a 21-day treatment of heat stress, heat stress with supplementation, pair-fed to heat stress, Pair-fed to heat stress with supplementation. Data are reported as means ± SEM. Statistical differences were accepted as significant at $P<0.05$ or a tendency at $P<0.10$.

Chapter 6 Summary

Muscle is a dynamic and adaptive tissue that responds to signals from its environment. Postnatal muscle growth occurs mainly through hypertrophy of existing fibers. Fractional growth during the neonatal period occurs at the highest rate as a percentage of body weight, requiring efficient satellite cell activity to facilitate the increased protein demand. Delay or absence of satellite cell activity leads to impaired growth potential of the animal. Neonatal pigs require sufficient nutrients to allow for this enhanced growth, and nutrient restriction can have lasting consequences on growth potential. Heat stress alters digestion and feed intake, causing changes in nutrient availability in muscle. Nutritional interventions have also been used as a strategy to improve pig growth performance during heat stress. Heat stress alters the metabolism of pigs resulting in decreased growth efficiency. The aim of this dissertation was to investigate nutritional interventions on growth performance of pigs.

Dietary calcium phosphate inclusion levels in the diet did not have a significant effect on growth of neonatal pigs; however, these levels influence satellite cell activity. Dietary calcium phosphate deficiency reduced satellite cell proliferation and fusion rates indicating impaired function. This reduction in function can lead to diminished muscle growth and repair later in life. Adequate dietary calcium phosphate results in the greatest satellite cell proliferation and fusion rates, which may be mediated through an increase in the expression of differentiation enhancing miR-206.

Altering dietary protein inclusion levels in the diet had the greatest impact on growth of the three neonatal nutritional studies. Growth rates increased with increasing protein levels. This resulted in increased body weight in excess protein pigs. Diets deficient in protein were detrimental to growth, and lead to an upregulation of protein degradation genes. Proliferation was not affected

by protein level; however, fusion index was altered resulting in the highest fusion rates in satellite cells from adequate and deficient protein pigs early in differentiation. Fusion rates initially were the lowest in satellite cells from excess protein diets; however, by day 3 rates were recovered and not different from adequate satellite cell fusion rates. Correspondingly, the differentiation promoting miR-1 was upregulated in excess satellite cells at day 0 which may have caused the increased fusion rates by day 3.

Dietary energy did not influence growth in neonatal pigs; however, it did impact satellite cell function. Fusion index was highest in satellite cells from deficient energy diets which corresponds with deficient energy diets having the highest expression of miR-1. During differentiation, adequate diets had the lowest fusion index at day 0 and high expression of miR-1 which lead to an increase in fusion index by day 3.

Nutritional interventions may be a cost-effective way to improve growth during heat stress. Consequences of heat stress include altered growth, metabolism, and metabolic flexibility. Supplementing with an artificial sweetener and capsicum oleoresin helped to maintain metabolic flexibility and improve feed efficiency during heat stress. These findings suggest that this supplement may improve the health of heat stressed animals and allow them to better cope, and ultimately encouraging recovered growth rates.

Nutrient restriction can lead to a reduction in growth and satellite cell function resulting in diminished muscle growth and repair later in life, however it is not known if these effects are sustained and for what duration. Future studies should seek to investigate the long-term effects of these nutritional interventions during the neonatal period in order to optimize growth. It appears from these data that micro-RNA play a key role in the effects nutritional status has on satellite cell function. Future studies are necessary to determine how miRNAs are influenced by nutrition.

Furthermore, additional research should seek to determine if supplementing with artificial sweetener and capsicum oleoresin can further improve animal health and growth depending on the length and severity of heat stress and the duration of supplementation.