Defining the role of mitochondria in fresh meat quality development

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ABSTRACT

During postmortem metabolism, hydrogen ions accumulate in the muscle and gradually lower the pH from 7.2 to an ultimate pH near 5.6. The ultimate pH of meat is widely valued as an indicator of fresh meat quality as it directly affects the quality characteristics of color, texture, and water holding capacity. Therefore, our research was conducted to identify the processes responsible for determining ultimate pH. Pigs harboring the AMPKγ3^{R200Q} mutation produce meat with extremely low ultimate pH (pH ~ 5.3) that is detrimental to quality. This phenomenon is often attributed to a greater glycogen content in muscle from the mutant pigs compared to wild-type pigs. However, our research indicated that greater glycolytic flux in muscle from these pigs causes a lower ultimate pH rather than greater tissue glycogen deposition. On the other hand, however, AMPKγ3^{R200Q} pigs contain more mitochondria and retain greater oxidative capacity. Hence, we hypothesized that mitochondria may contribute to the lower ultimate pH in muscle of these pigs. To test our hypothesis, isolated mitochondria were incorporated into an *in vitro* system the mimics postmortem glycolysis. Mitochondria enhanced glycolytic flux and pH decline in the *in vitro* system similar to that of AMPKγ3^{R200Q} pigs. After a series of experiments, we found that the causative agent for enhanced glycolytic flux is a soluble mitochondrial protein. In other experiments, mitochondrial F_1-ATPase was found to be responsible for the majority of this effect, principally through promoting greater ATP hydrolysis at lower pH values, thereby allowing for greater flux through glycolysis. These data suggest that variations in ultimate pH may be more thoroughly explained and predicted by the abundance of mitochondria.

Broiler *pectoralis major* muscle, which is a highly glycolytic muscle, possesses high ultimate pH (pH ~ 5.9) compared to pork and beef. We postulated that rapid carcass chilling reduces the flux through glycolysis, thereby causing premature termination of postmortem metabolism. Yet, chilling was only partially responsible for the high ultimate pH of *pectoralis major* muscle. However, we showed that *pectoralis major* of broiler chicken exhibits lower phosphofructokinase-1 activity compared to porcine *longissimus lumborum* muscle, which limits the flux through glycolysis.

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GENERAL AUDIENCE ABSTRACT

Consumer demand for high quality meat has increased dramatically over the past two decades. In order to meet this demand, it is crucial to understand factors that control the development of fresh meat quality characteristics. Consumers purchase meat based on color, but repeat purchases are also influenced by meat freshness, texture, and juiciness. These quality attributes develop after the animal has been harvested during the conversion of muscle to meat through a series of biochemical reactions. This conversion results in muscle acidification (pH decline) caused by the degradation of stored muscle energy that acidifies muscle. Normally, the muscle pH drops from a neutral value in living muscle to an acid value (~5.6) in fresh meat. In some cases, however, excessive acidification occurs and this can dramatically impact fresh meat quality characteristics. Our research program focuses on the mechanisms responsible for this extended acidification. To that end, we use mutant pigs known as RN^- that produce meat with extremely low ultimate pH known as “acid meat”. While most believe that the extended pH decline in muscle of these pigs is a function of elevated energy in the muscle prior to harvesting, we showed that theses pigs have different muscle prior to harvest and this difference cause increased acidification during the transformation of muscle to meat. To investigate this issue further, we also examined the contribution of mitochondria (the powerhouse of the cell) to this process mainly because muscle from the RN^- pigs containing around 50% more mitochondria compared to normal pigs. Curiously, we have shown mitochondria participate in this process. Because mitochondria require oxygen to function and harvesting animals disrupts oxygen delivery to the muscle, mitochondria were considered irrelevant to the development of meat quality characteristics. Our

studies have definitively proved that mitochondria can contribute to meat quality and may be key in improving fresh meat quality.

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Chapter 1 – The conversion of muscle to meat

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Introduction

During the postmortem period, a complex cascade of energetic, biochemical, and physical changes take place in the muscle that results in its conversion to meat. The process begins shortly after harvest when many of the homeostatic mechanisms of the animal are disrupted. As the animal succumbs to exsanguination and resulting anoxia, skeletal muscle continues to synthesize and utilize adenosine triphosphate (ATP) in a futile attempt to sustain cellular homeostasis. Once oxygen is depleted from the muscle, glycogen and high-energy phosphate compounds present in the muscle at the time of death are anaerobically metabolized for the sole purpose of ATP production. Anaerobic metabolism is significantly less efficient at generating ATP than aerobic metabolism. As a result, the rate of ATP hydrolysis exceeds its generation, which triggers the onset of rigor mortis (Latin for "stiffness of death"). As postmortem metabolism proceeds, the muscle gradually loses the ability to generate ATP and eventually all ATP is depleted. In the absence of ATP, myosin binds irreversibly to actin, leading to the completion of rigor mortis and the loss of muscle excitability and extensibility. The completion of rigor mortis occurs at 1-12 h postmortem depending mainly on the species, muscle fiber type, and ante- and postmortem conditions. During postmortem storage (aging), the proteolytic degradation of cytoskeletal proteins causes the loss of muscle structural integrity, and thus a decrease in muscle tension (resolution of rigor mortis).

Another significant change occurs in postmortem muscle under anaerobic conditions, acidification. The end product of postmortem glycolysis and ATP hydrolysis, lactate and hydrogen ions (H^+), respectively, accumulate in the muscle due to the lack of an effective elimination mechanism. As a result, muscle pH gradually declines from around 7.2 in living tissue to an ultimate pH near 5.6. The rate and extent of postmortem metabolism significantly

influence the development of meat quality attributes. Hastened, extended, or insufficient postmortem pH decline adversely influences meat color, texture, and water-holding capacity. Factors such as environmental conditions and pre- and post-slaughter handling can significantly alter postmortem pH decline. Therefore, understanding the factors that control the rate and extent of postmortem metabolism can influence the probability of producing high quality product.

Postmortem metabolism

ATP homeostasis

Skeletal muscle is a highly dynamic tissue and exhibits a remarkable ability to change its metabolic capacity to meet the demands imposed on it. The energy requirement of skeletal muscle remains relatively low under resting conditions, but can quickly increase its capacity 100 fold during periods of intense activity. The nucleotide coenzyme ATP is the primary currency of cellular energy transfer. Energy liberated from the hydrolysis of the energy-rich phosphate bonds of ATP (reaction 1) is used to perform cellular functions. This includes muscle contraction, active ion transport, cell signaling, and biosynthesis of macromolecules. Given these vital functions, the energy capacity of muscle tissue must maintain ATP homeostasis over a wide range of cellular challenges and circumstances. Yet, skeletal muscle stores a limited amount of ATP (5-8 μmol/g of muscle tissue); only enough to maintain muscle energetic demands for no longer than a few seconds of high intensity work. Therefore, ATP must be continuously generated by other mechanisms that involve the catabolism of stored energy compounds such as carbohydrates and lipids.

$$ATP + H_2O \xrightarrow{ATPase} ADP + P_i + H^+ + energy + heat \quad (1)$$

Many of these same cellular functions operate seamlessly during the conversion of muscle to meat and essentially involve the synthesis, hydrolysis, and availability of ATP during a

cataclysmic event for the tissue death. There are three major energetic pathways by which muscle generates ATP: the phosphagen system, glycolysis, and oxidative phosphorylation. Understanding the role of each pathway in postmortem metabolism is fundamental to understanding the process of converting muscle to meat.

The phosphagen system

During early postmortem metabolism, muscle ATP concentration remains stable through the utilization of a high-energy phosphate compound known as phosphocreatine (PCr). Phosphocreatine (or creatine phosphate) serves as an immediate energy source that rapidly buffers ATP levels during high-energy demands. The enzyme creatine kinase (CK) catalyzes the reversible transfer of an inorganic phosphate (P_i) from PCr to adenosine diphosphate (ADP) to form ATP and creatine. While under resting conditions the reaction favors the formation of PCr, during high-ATP demands PCr is degraded and the free energy released is used to regenerate ATP from ADP. Because muscle stores of PCr are also limited (~ 25 μmol/g of muscle tissue), PCr can only sustain postmortem cellular ATP for a brief period. Creatine kinase is one of the three enzymes that comprise the so-called phosphagen system, with the other two enzymes being adenylate kinase (AK) and adenosine monophosphate deaminase (AMPD).

As soon as the majority of PCr has been degraded, the rate of ATP hydrolysis has likely exceeded the rate of resynthesis, leading to an excessive formation of ADP. This activates the enzyme AK, which buffers the drop in ATP by converting two molecules of ADP into an ATP and AMP. Subsequently, AMP is irreversibly deaminated by the enzyme AMPD to form inosine monophosphate (IMP), which accumulates in the muscle. The AMPD reaction is important to shift the equilibrium of the AK reaction in the direction of ATP formation. However, deamination of AMP is responsible for the drop in the total adenine nucleotide pool (ATP, ADP,

and AMP) because IMP is unable to contribute to ATP synthesis.

It is important to note that the net production of H^+ by the phosphagen system during postmortem metabolism is zero. Protons released through the hydrolysis of ATP generated by the phosphagen system are in turn consumed by the CK and AMPD reactions. Nonetheless, metabolites produced by the phosphagen system (AMP, ADP, P_i) function as activators for rate-limiting enzymes in the glycolytic pathway. Using an in *vitro model* system, England et al. (2015) found that increased AMP concentration through reducing AMPD activity increased the rate and the extent of glycolysis. On the other hand, alteration to the antemortem concentration of total muscle creatine pool (PCr + creatine) was associated with higher ultimate pH and enhancements in meat quality (Scheffler et al., 2013a).

Glycogenolysis and glycolysis

The capacity of the phosphagen system to maintain postmortem ATP homeostasis is limited to the available PCr and adenine nucleotides at the time of harvest. As PCr concentration drops below 4 μmol/g of muscle, the catabolism of muscle glycogen through glycogenolysis (glycogen degradation) and glycolysis becomes the dominant pathway for ATP production (Bendall, 1973).

Glycogen is the predominant storage form of carbohydrate in skeletal muscle, and represents 1-2% of the total muscle mass. It is a highly branched polymer of glucose residues held together linearly by α-1,4-glycoidic bonds. At every 8-12 residues, a branch is formed by α-1,6-glycosidic bonds. Branching is important because it increases the solubility of glycogen and the number of non-reducing terminal glucose residues (glucose with a free OH group at C-4), thus increasing the number of sites accessible to the enzymes involved in glycogen degradation. Glycogen exists in the form of granules (10-40 nm) in the sarcoplasm of the muscle fiber that contains the enzymes needed for glycogenolysis.

Complete degradation of glycogen requires a combined action of two enzymes: glycogen phosphorylase (GP) and glycogen debranching enzyme (GDE). GP catalyzes the sequential phosphorolytic cleavage of the α-1,4-linkages at the non-reducing ends of the glycogen chains. Inorganic phosphate splits the glycosidic bond between the terminal glucose moiety and the adjacent one to yield glucose 1-phoshate, thus leaving the glycogen chain one glucose shorter (reaction 2). Because glycogen phosphorylase is incapable of cleaving α-1,6-linkages at the branch points and indeed stops cleaving four residues away, the action of another enzyme is required. GDE has two distinctive catalytic activities: transferase and α-1,6-glucosidase. The transferase exposes the α-1,6-linkages by transferring the terminal three glycosyl residues to an adjacent chain to extend it. The last glucose residue remaining at the branch point is hydrolyzed by α-1,6-glucosidase and released as a free glucose molecule. Glucose 1-phosphate molecules resulting from the action of GP can be readily converted to glucose 6-phosphate by phosphoglucomutase (PGM) (reaction 3). Free glucose molecules are either converted by the enzyme hexokinase to glucose 6-phosphate (reaction 4) or accumulate in the muscle postmortem.

$$\text{Glycogen}_n + P_i \xrightarrow{GP} \text{Glycogen}_{n-1} + \text{Glucose 1-phosphate} \qquad (2)$$

$$\text{Glucose 1-phosphate} \xrightarrow{PGM} \text{Glucose 6-phosphate} \qquad (3)$$

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{Glucose 6-phoshate} + \text{ADP} + H^+ \qquad (4)$$

Glucose 6-phosphate obtained through glycogenolysis, in addition to the glucose 6-phosphate stored in the muscle, can directly enter the glycolytic pathway (glycolysis): a sequence

of ten reactions all of which occur in the sarcoplasm of the muscle fiber. During glycolysis, each six-carbon glucose moiety of glycogen is mobilized into two three-carbon pyruvate molecules, in addition to a net yield of three molecules of ATP, two molecules of NADH, one H^+ and two water molecules (reaction 5):

$$\text{Glycogen}_n + 3\ \text{ADP} + 3\ P_i + 2\ \text{NAD}^+ \longrightarrow \text{Glycogen}_{n-1} + 2\ \text{pyruvate} + 3\ \text{ATP} + 2\ \text{NADH} + H^+ + 2\ H_2O \quad (5)$$

Under anaerobic conditions, pyruvate is reduced to lactate by the enzyme lactate dehydrogenase (LDH) (reaction 6). This reaction is crucial to regenerate NAD^+ that is required for the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) reaction, thereby allowing glycolysis to continue under anaerobic conditions. Due to the absence of circulation, lactate accumulates in the muscle postmortem. Yet, lactate itself is not responsible for the drop in pH. Indeed, the LDH reaction functions as a buffer by consuming one H^+ from the system for every pyruvate molecule converted to lactate (reaction 6). Consequently, when glycolysis is coupled to lactate formation, a net decrease of one H^+ occurs for each glucose moiety mobilized (reaction 7). Instead, the hydrolysis of the ATP yields H^+ (reaction 1) that accumulates in the muscle postmortem and lowers the pH. Honikel and Hamm (1974) indicated that the hydrolysis of ATP generated through postmortem glycolysis is responsible for 90% of the H^+, while the remaining 10% originated from the hydrolysis of ATP present in the muscle at the time of death. When glycolysis is combined with lactate formation and ATP hydrolysis, a net of two lactate and two H^+ are produced (reaction 8). Because glycolysis generates H^+ and lactate at 1:1 ratio, a negative linear relationship is often observed when postmortem muscle pH values are plotted against

lactate values measured at the same time, making lactate a good indicator for the extent of postmortem metabolism.

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+ \quad (6)$$

$$\text{Glycogen}_n + 3\ \text{ADP} + 3\ \text{P}_\text{i} + \text{H}^+ \longrightarrow \text{Glycogen}_{n-1} + 2\ \text{lactate} + 3\ \text{ATP} + 2\ \text{H}_2\text{O} \quad (7)$$

$$\text{Glycogen}_n \longrightarrow \text{Glycogen}_{n-1} + 2\ \text{lactate} + 2\ \text{H}^+ \quad (8)$$

The flux through the glycolytic pathway is regulated by three rate-limiting enzymes: GP, phosphofructokinase-1 (PFK-1), and pyruvate kinase (PK). The concentration of allosteric regulators, feedback mechanisms (end-product regulation), and covalent modification (phosphorylation or dephosphorylation) have been shown to regulate the activities of the aforementioned enzymes. These regulatory mechanisms allow the glycolytic pathway to accommodate changes for energy demand by increasing or decreasing the activities of the enzyme. Thus, understanding mechanisms controlling the flux through the glycolytic pathway is essential to understand postmortem glycolysis and ultimately fresh meat quality.

GP, the key enzyme in glycogenolysis, exists in two interconvertible forms: the less active b (dephosphorylated) form and the more active (phosphorylated) a form. Phosphorylase b is allosterically activated by AMP, IMP and by high levels of P_i, and inhibited by ATP and glucose 6-phosphate. In response to AMP, phosphorylase b undergoes conformational changes that enhance the enzyme-substrate binding affinity (lowers the K_m). Phosphorylation of the Ser^{14}

residue in phosphorylase b by the enzyme phosphorylase kinase converts it to phosphorylase a, the fully active form independent of AMP stimulation.

PFK-1 is the key regulatory enzyme of glycolysis and one of the most tightly regulated enzymes in metabolism. PFK-1 catalyzes the irreversible phosphorylation of fructose 6-phosphate to fructose 1,6-phosphate; a reaction that consumes one molecule of ATP. The activity of PFK-1 is regulated by allosteric regulators, pH, and formation of oligomer structure. PFK-1 is allosterically stimulated by different ligands including AMP, ADP, fructose 2,6-bisphosphate, while ATP, PCr, and citrate act as inhibitors. The enzyme exists in different oligomeric structures including dimers, tetramers, and larger multimers. PFK-1 dimers exhibit minimal catalytic activity, while tetramers (dimer of dimers) and higher aggregates are fully active. Low pH and high concentration of allosteric inhibitors favor the formation of dimers, whereas higher pH and greater concentration of allosteric activators stabilize the tetramer form.

The last rate-limiting enzyme of glycolysis is PK, which irreversibly catalyzes the conversion of phosphoenolpyruvate to pyruvate. This reaction is coupled to ATP synthesis and accounts for two ATP molecules produced by postmortem glycolysis for each molecule of glucose metabolized. PK is allosterically activated by AMP and the upstream intermediate fructose 1,6-bisphosphate and inhibited by ATP and acetyl-CoA.

The role of mitochondria

Mitochondria are often referred as the powerhouse of the cell because it is the place where the majority of cellular ATP is produced through a process called oxidative phosphorylation. Mitochondria consist of two membranes (inner and outer), hence creating two separate mitochondrial compartments: the intermembrane space between the outer and the inner membrane and the matrix, which is bound by the highly convoluted inner membrane. The outer

membrane is highly porous and permeable to most ions and small molecules. In contrast, the inner membrane is impermeable to the passage of nearly all ions and molecules, unless mediated by a membrane transport protein. The matrix is the site for the tricarboxylic acid cycle, where energy liberated from the oxidation of acetyl-CoA is conserved in the structure of the reduced coenzymes NADH and $FADH_2$. Embedded in the inner mitochondrial membrane are components of the electron transport chains. During cellular respiration, electrons are released from NADH and $FADH_2$ to the electron transport chain and finally to O_2 to yield H_2O. The movement of electrons through the electron transport chain is coupled with the pumping of H^+ from the matrix to the intermembrane space. These protons build up and create an electrochemical potential gradient across the inner membrane (proton motive force). The flow back of protons into the matrix down their electrochemical gradient through the F_1F_0 ATP synthase drives the synthesis of ATP.

The cessation of oxygen delivery to the muscle following exsanguination impedes the mitochondria's ability to produce ATP through oxidative phosphorylation. Therefore, mitochondria are often considered irrelevant to the process of converting muscle to meat. However, mitochondria do not "die" immediately postmortem, rather they maintain functionality and structural integrity for several hours (Tang et al., 2005). Therefore, mitochondria may influence postmortem metabolism by altering the biochemical and the energetic properties of the muscle. In addition, mitochondria are involved in cellular Ca^{2+} homeostasis, apoptosis (programmed cell death), and meat color stability.

As the oxygen supply is removed due to lack of blood circulation, skeletal muscle utilizes oxygen bound to myoglobin for energy production. Because oxidative phosphorylation generates 10 times more ATP than anaerobic glycolysis (30 vs 3, respectively), aerobic activity for even

short period of time postmortem would significantly improve maintenance of ATP levels. Pösö and Puolanne (2005) estimated the amount of postmortem aerobic ATP production using oxygen stored in the muscle to be between 0.4 to 6 μmol/g of muscle, depending on animal age, species, and muscle fiber type. Recently, we showed that inclusion of functioning mitochondria to an *in vitro* model mimics postmortem metabolism reduces the rate of ATP loss (Scheffler et al., 2015). Because maintaining adenine nucleotides for longer periods of time postmortem can extend glycolysis (Greaser, 1986), differences in mitochondrial oxidative capacity between muscles could influence the onset and the extent of anaerobic metabolism postmortem. While a promising step forward, further investigation is necessary to improve our understanding of the role of mitochondria in postmortem metabolism.

ATP depletion under postmortem anoxic conditions impairs sarcoplasmic reticulum Ca^{2+} uptake by the Ca^{2+} ATPase pump, resulting in a cytosolic Ca^{2+} overload. As a part of Ca^{2+} buffering system, mitochondria sequester large quantities of Ca^{2+} in an attempt to maintain cytosolic Ca^{2+} homeostasis. Excessive mitochondrial Ca^{2+} loading increases the formation of reactive oxygen species (ROS), which induce oxidative stress. High levels of ROS and Ca^{2+} stimulate mitochondrial permeability transition pore (mPTP) opening. As a result, apoptosis-inducing proteins like cytochrome c are released into the cytosol and activate the downstream caspases leading to cell death. In addition, the opening of mPTP is associated with the loss of the inner mitochondrial membrane potential. To avoid the collapse of the proton motive force, the F_1F_0 ATP synthase switches roles and commits "treason" by coupling the pumping of H^+ across the inner membrane to ATP hydrolysis (St-Pierre et al., 2000). The energetic need for this function is obtained from ATP produced by glycolysis, which in turn exacerbates cellular energy deficit. The ATP-driven proton pumping activity of ATP synthase could increase the rate of ATP

hydrolysis and subsequently postmortem metabolism, which in turn, adversely influence meat quality.

The factors controlling the rate of postmortem metabolism

The rate of pH decline during the conversion of muscle to meat reflects the intensity of postmortem metabolism. Typically, pH gradually declines from around 7.2 to 5.8 within 8 h postmortem, with an ultimate pH of about 5.6 achieved at 24 h. The rate of postmortem pH decline is influenced by many factors including: species, genetics, muscle fiber type, and pre- and postmortem handling conditions. In general, the rate of postmortem pH decline differs among meat species in the following order: poultry > pork > beef > lamb. Scopes (1974) concluded that the rate of ATP hydrolysis by muscle ATPases drives the rate of postmortem metabolism. In addition, he indicated that mechanisms controlling the flux through the rate-limiting enzymes of the glycolytic pathway must be determined by activity of ATPases. Several enzyme systems present in the muscle such as myosin ATPase, sarcoplasmic reticulum Ca^{2+} ATPase, Na^{+}/K^{+} ATPase, and mitochondrial ATPase require the hydrolysis of ATP in order to perform cellular functions. Because myosin is the most abundant protein in the muscle, myosin ATPase is considered to be the major ATPase responsible for ATP hydrolysis postmortem (Hamm et al., 1973). ATP splitting rate by muscle ATPases at 38 °C is approximately 0.5 μmol / min per g of muscle (Scopes, 1973). The rate of ATP turnover decreases with decreasing temperature from 38 to 15 °C, however, ATP depletion rate rises again around 0 °C (Newbold and Scopes, 1967; Bendall, 1973). At lower temperatures, Ca^{2+} release from the sarcoplasmic reticulum is enhanced while Ca^{2+} sequestering is impaired, thereby increasing the cytosolic concentration of Ca^{2+}.

The rate of ATP hydrolysis dramatically increases as cytosolic Ca^{2+} rises. Under high cytosolic concentration of Ca^{2+}, the inhibition of muscle contraction by the troponin-tropomyosin complex is removed, allowing the interaction between myosin cross-bridges and adjacent actin active sites. Approximately 10-fold increases in semitendinosus myofibrillar ATPase activity was observed when the level of Ca^{2+} increases from pCa^{7} to pCa^{6} or less (Bowker et al., 2004). Additionally, Ca^{2+} ATPase catalyzes the breakdown of ATP in an attempt to sequester Ca^{2+} back into the sarcoplasmic reticulum, which further increases the rate of ATP hydrolysis. Further, high sarcoplasmic Ca^{2+} can also increase the rate of postmortem glycolysis through the activation phosphorylase kinase by binding to its calmodulin subunits. Activated phosphorylase kinase subsequently phosphorylates and activates the enzyme GP. Thus, Ca^{2+} plays a significant role on defining the rate of postmortem metabolism. Passing an electric current through the carcass early postmortem (electrical stimulation) accelerates postmortem metabolism by the massive liberation of Ca^{2+} from the sarcoplasm reticulum. Electrical stimulation has been widely used in red meat (mainly beef and lamb) to accelerate ATP depletion and the development of early rigor mortis. This process reduces the incidence of cold shortening, in addition to improving meat tenderness, color, and sensory characteristics (Nazli et al., 2010; Cetin et al., 2012).

The factors controlling the extent of postmortem metabolism

The extent of postmortem acidification is a key factor determining meat quality development as it influences meat color, texture, water-holding capacity and shelf life. The normal ultimate pH value of meat in most meat species ranges between 5.5-5.7 and meat within this range possesses the most desirable quality characteristics. Meat at pH 6.0 or greater appears darker in color and has a shorter shelf life, while meat of pH < 5.4 has a pale color, lower water holding capacity, reduced protein extractability, and poor processing yield. Therefore, ultimate pH is widely

regarded as an indicator of fresh meat quality. The ultimate pH of meat is a function of many factors. However none of the factors are sufficient in predicting more than 50% of the variation in ultimate pH (reviewed by Van Laack et al., 2001a). Therefore, understanding factors determine the ultimate pH is key to minimize variations in meat quality.

Recall, anaerobic mobilization of glycogen during postmortem metabolism drives pH decline. Therefore, the extent of postmortem metabolism would be expected to be a function of muscle glycogen content at the time of slaughter. In other words, the pH of the muscle should continue dropping as long as glycogen is available in the muscle. This, however, is not the case. Postmortem glycolysis usually stops in the presence of residual glycogen in the muscle of certain species. The relation between glycogen content and ultimate pH is curvilinear instead of linear. Ultimate pH decreases as glycogen increases until a plateau is reached. Henckel et al. (2002) found that the extent of pH decline is determined by glycogen content, provided glycogen levels fall between 0 and 53 μmol/g of muscle. Any further increase in glycogen levels beyond 53 μmol/g is not associated with further decline in pH. Van Laack et al. (2001a) showed that differences in muscle glycogen accounts for less than 40% of the variation in ultimate pH.

The cessation of postmortem metabolism in the presence of residual glycogen and glycolytic intermediates suggest that other biochemical mechanisms influence determination of ultimate pH. Two hypotheses have been proposed, pH-mediated inactivation of one or more glycolytic enzyme and/or the loss of adenine nucleotides through the phosphagen system (Kastenschmidt et al., 1968; Bendall, 1973). England et al. (2014) indicated that PFK-1 begins to lose activity around pH 5.9 and becomes completely inactive at pH 5.5, while other enzymes including GP and PK maintain functionality at pH 5.5. The authors suggested that PFK-1 may function as a substrate ‘gate’. Essentially, glycogen can be converted to lactate and H^+ provided

PFK-1 maintains activity. As pH declines, the ability of this process to occur becomes limiting and postmortem glycolysis eventually stops. However, PFK-1 inactivation may not explain why some muscles produce meat with a high ultimate pH (pH > 5.9) in the presence of residual glycogen. Instead, the complete conversion of adenosine nucleotides to IMP by AMPD arrests glycolysis (England et al., 2016). Anaerobic glycolysis and phosphagen system work synergistically in an attempt to maintain cellular ATP homeostasis postmortem. While glycolysis preserves adenine nucleotides by recycling ADP back to ATP, the phosphagen system is responsible for the loss of adenine nucleotides. Oxidative (red) muscle has less glycolytic enzymes abundance and activity (less glycolytic capacity) and thereby a slower rate of postmortem glycolysis compared to glycolytic (white) muscle. Therefore, oxidative muscle is unable to "keep up" with the phosphagen system, resulting in the loss of nucleotides and the cessation of metabolism while glycogen is still available (England et al., 2016). The activity of AMPD can influence the two aforementioned mechanisms responsible for postmortem termination. Lower AMPD activity increases AMP concentration, which in turn activates the rate-limiting enzymes of glycogenolysis and glycolysis (England et al., 2015). Thus leading to an increase flux through PFK-1 and a lower the pH. Moreover, lower AMPD activity may delay the loss of adenine nucleotides and therefore extend metabolism (Greaser, 1986).

Muscle acidification during the conversion of muscle to meat is the net result of total H^+ produced postmortem minus those bound and neutralized by buffering systems in the muscle. Nearly 50% of the muscle buffering capacity is due to myofibril proteins, while phosphate compounds, histidine containing dipeptides carnosine and anserine, and lactate contributed to the other half (Honikel and Hamm, 1974). The buffering capacity of skeletal muscle among meat species varies between 40 to 60 μmol $H^+ * pH^{-1} * g^{-1}$, depending on the type of muscle. Typically,

buffering capacity is greater in white muscles than those of red muscles. This is likely due to the greater contents of inorganic phosphate and carnosine. Van Laack et al. (2001b) suggested that buffering capacity might explain differences in ultimate pH between muscles with similar lactate levels. While differences in buffering capacity may help to explain the extent of pH decline, buffering capacity does not appear to be a major determinant of ultimate pH (Puolanne and Kivikari, 2000).

In summary, the extent of postmortem metabolism is dictated by the glycogen content of the muscle as long as levels are ≤ 53 μmol/g. If glycogen is > 53 μmol/g, PFK-1 brackets ultimate pH into a fairly consistent range near pH 5.6. A unique situation exists in some oxidative muscle, where metabolism is terminated while PFK-1 presumably still functioning. While most believe glycogen depletion arrests pH decline, our data suggest glycogen content alone is unable to explain the full limitation in pH decline. Rather, the loss of adenosine nucleotides arrest glycolysis (England et al., 2016). Finally, buffering capacity and the activity of AMPD help explain variations in ultimate pH (pH 5.4–5.8) in the same muscle between animals from genetically similar background.

Abnormal postmortem metabolism

Pale, soft, and exudative (PSE) meat

Pale, soft, and exudative (PSE) is the term used to describe meat with abnormally light color, soft texture, and impaired ability to hold water. This defective condition is caused by excessively rapid metabolism immediately following slaughter when carcass temperature is still elevated. The pH of PSE meat drops to a value around the ultimate pH of muscle within the first hour postmortem. The combined action of low pH and high temperature leads to an extreme denaturation of many sarcoplasmic and myofibrillar proteins in the muscle. It should be noted

that the rate of pH decline associated with PSE meat may not necessarily produce meat with a lower ultimate pH.

Intensive selection for high body weight and meat yield in pigs and poultry shifts muscle fiber towards a more glycolytic fiber type. Typically, glycolytic muscle of pigs and poultry exhibits rapid glycolysis and thus greater susceptibility for PSE. Due to the high incidence (up to 40%), PSE has become one of the biggest challenges facing the meat industry worldwide, in particular pork and poultry (Petracci et al., 2009).

The development of PSE condition is mainly associated with genetic factors and pre-slaughter stressors including: environmental stressors, improper handling, and mixing with unfamiliar animals. Stressful conditions activate the sympathetic nervous system, which in turn prompts the secretion of epinephrine from the adrenal medulla. Once released, epinephrine binds to β-adrenergic receptors on skeletal muscle, triggering a signaling cascade that stimulate glycogenolysis to sustain high glucose levels. The stimulation of β-adrenergic receptors activates cAMP-dependent protein kinase A, which successively phosphorylates and activates phosphorylase kinase. Activated phosphorylase kinase phosphorylates the less active form glycogen phosphorylase b to the more active a form, enhancing glycogen degradation. In addition, protein kinase A phosphorylates and activates ryanodine receptor type 1 RYR1 (Ca^{2+} releasing channel), causing rapid release of Ca^{2+} from the sarcoplasmic reticulum into the cytosol. High amplitude Ca^{2+} release accelerates glycolysis by increasing muscle ATPase activities and the flux through glycolysis.

In pigs, PSE meat is often associated with porcine stress syndrome (PSS), a condition analogous to human malignant hyperthermia. Pigs with PSS lack the ability to adapt to environmental stressors, which leads to severe muscle contractions, a rapid increase in body

temperature, cardiac arrest and death (reviewed by Ball and Johnson, 1993). PSS is greatly induced by a mutation known as halothane mutation (HAL or HAL-1843), a single base substitution (C1843 to T) mutation in the RYR1 gene (halothane gene). The mutation results in a replacement of arginine by cysteine at resides 615 at the protein level. Halothane (HAL) gas was used as screening method to identify animals susceptible to PSS, and since then the genetic component has been referred to as the HAL gene. HAL positive pigs are hypersensitive to agents that stimulate opening, leading to an excessive release of Ca^{2+} into the sarcoplasm.

Because of the apparent similarity of PSE condition between pigs and poultry, a genetic component was thought to be responsible for with PSE susceptibility in poultry. However, to date, evidence for genetic basis of PSE poultry is limited. Oda et al. (2009) found lower RYR β-isoform (β-RYR) gene expression in PSE chicken breast compared to non-PSE. These finding suggests that the differential expression of β-RYR could contribute to the development of PSE in chicken. PSE in poultry has been associated with ante-mortem stress, and in particular, heat stress that can causes accelerated postmortem metabolism. Improper chilling of carcasses postmortem can also develop PSE conditions in poultry. Minimizing stress during handling and transport while optimizing chilling conditions can reduce the incidence of PSE in poultry.

Acid meat

Another porcine genetic mutation that often leads to abnormally low ultimate pH meat (acid meat, pH < 5.4) is AMPKγ3^{R200Q}, also known as, Rendement Napole (RN^{-}) mutation. AMPKγ3^{R200Q} is a point mutation in the PRKAG3 gene that encodes the γ3 regulatory subunit of AMP-activated protein kinase (AMPK), resulting in a single amino acid substitution at residue 200 from arginine to glutamine (Milan et al., 2000). This mutation is most notably found in Hampshire pigs and renders a constitutively active AMPK in muscle tissues. The gain-in

function mutation results in approximately 100% increase in glycogen content and enhanced mitochondrial oxidative capacity in glycolytic muscles.

The extended postmortem pH decline in AMPKγ3^{R200Q} pigs is characterized by normal rate, but continues to drop for a longer time. The resulting ultimate pH is near the isoelectric point (p*I*), or the pH at which the positive and negative charges of muscle protein side groups are approximately equal (pH 5.1-5.2), thereby reduces the ability of muscle to bind water. Low ultimate pH associated with AMPKγ3^{R200Q} mutation adversely influences meat water holding capacity, protein content and functionality, and processing yield. The abnormally low ultimate pH of AMPKγ3^{R200Q} mutant pigs is usually attributed to the fact that these pigs deposit great muscle glycogen. Yet, muscle from AMPKγ3^{R200Q} pigs accumulate similar lactate levels at 24 h postmortem to that of muscle from wild-type pigs. Scheffler et al. (2013b) concluded that glycogen content does not have a direct effect on the extent of postmortem pH decline. Recently, we showed that greater glycolytic flux coupled with a lower postmortem buffering capacity explains the lower ultimate pH of meat from AMPKγ3^{R200Q} pigs (Matarneh et al., 2015). However, reasons beyond the greater flux in the AMPKγ3^{R200Q} pigs are not well understood. Scheffler et al. (2011) proposed that other metabolic properties (i.e. mitochondria, PCr, and glycolytic enzymes content and activity) could play participating roles in determination of ultimate pH in the AMPKγ3^{R200Q} mutant pigs. However, these factors might work through the activation of glycogenolysis and glycolysis as indicated by the greater glycogen degradation and net lactate accumulation in the AMPKγ3^{R200Q} pigs (Matarneh et al., 2015). England et al. (2015) observed that activity and abundance of the enzyme AMPD is lower in the AMPKγ3^{R200Q} pigs, which results in greater AMP levels late postmortem. At present, both RN^{-} and HAL mutations have been eliminated from commercial swine industry and as such, RN^{-} and HAL-mediated

reductions in pork quality have been reduced dramatically. However, these two mutations remain valuable as models for the study of the rate and extent of postmortem pH decline.

Dark, firm, and dry meat

Dark, firm, and dry (DFD) or dark cutting is a meat quality defect observed predominantly in beef and to a leaser extent in lamb and pork. As the name implies, DFD meat is characterized by its abnormal dark color, firm texture, and dry sticky surface. This condition is a function of muscle glycogen deficiency and results from chronic exposure to pre-slaughter stresses. Inadequate glycogen leads to early termination of postmortem metabolism, and therefore limiting the drop in pH (ultimate pH > 6.0). Meat color is directly correlated to the ultimate pH; color progressively darkens as pH increases from 5.8 to 7.0. High ultimate pH minimizes meat pigment losses and denaturation, thereby increases light absorbance, which gives the meat darker appearance. Meat of elevated pH also exhibits higher water-holding capacity as the pH is further from the p*I*. This increases the negative charges on protein side groups available for binding water. Although the high water-holding capacity makes DFD meat a superior raw material for use in processed meat products, the difficulty to retail and the higher susceptibility to microbial spoilage makes DFD a major concern for beef industry in particular.

The exposure to prolonged antemortem stresses triggers glycogen degradation to meet the energy demands under stress. DFD conditions are developed when an animal fails to restore muscle glycogen reserves prior to slaughter. The repletion of muscle glycogen during the recovery period is typically a slow process, particularly in ruminants. This is usually attributed to the relatively low blood glucose level in ruminants as very little is absorbed from their gastrointestinal tract, and therefore the potential for greater incidence of DFD. McVeigh and Tarrant (1982) reported that glycogen restoration rate in beef *longissimus* muscle, following

epinephrine-induced glycogen depletion, was 1.5, 6.1, and 7.6 μmol/g per day for fasted, hay fed, and barely fed animals, respectively. The energy density of a bovine finishing diet can affect muscle glycogen content, an effect emphasized by the comparison between grain and grass fed cattle. When compared to grain fed cattle, pasture fed cattle have higher susceptibility to dark cutting. In general, feeding cattle a high energy diet (concentrate-based) increases the production of the volatile fatty acid propionate, a major precursor for hepatic gluconeogenesis, which in turn increases the capacity for glycogen deposition in the muscle (Daly et al., 1999).

Fresh meat quality

color

Color is considered the most important sensory attribute of fresh meat. This is largely because it is the first quality indicator used by consumers to evaluate the quality and the freshness of meat. Meat color varies according to the concentration and the redox state of heme pigments (Jeong et al., 2009; Sen et al., 2014) and myoglobin is considered to be the main heme pigment responsible for fresh meat color. Contribution of other heme proteins such as hemoglobin and cytochrome C is often considered negligible (reviewed by Mancini and Hunt, 2005; reviewed by Joo et al., 2013). In general, darker and redder meat is indicative for higher myoglobin content. Myoglobin content of muscle varies among different species and muscle fiber types, and generally increases concurrently with animal age (Jeong et al., 2009; reviewed by Joo et al., 2013). Myoglobin exists in three redox forms; oxymyoglobin, deoxymyoglobin and metmyoglobin, and the relative proportion of these determine the color of fresh meat (reviewed by Mancini and Hunt, 2005; Bjelanovic et al., 2013). Depending on oxygen availability, myoglobin can be readily alternate between its deoxy- and oxy- forms. In contrast, if the oxygen tension is low, myoglobin heme iron ($Mb\text{-}Fe^{+2}$) loses an electron and forms the oxidized ($Mb\text{-}Fe^{+3}$) brown undesirable pigment metmyoglobin (reviewed by Seideman and Cross, 1984).

Fresh meat color is significantly influenced by the rate and the extent of postmortem energy metabolism. Paler meat is associated with hastened or extended pH decline (pH < 5.4), while darker meat cuts are related to high ultimate pH (pH > 6.0) (reviewed by Scheffler and Gerrard, 2007). Low ultimate pH causes denaturation of the globin moiety of myoglobin, which exposes the heme portion, allowing for rapid oxygen dissociation from the heme and subsequent oxidation to metmyoglobin (reviewed by Seideman and Cross, 1984). Sugawara and Shikama (1980) studied the effect of pH on bovine myoglobin autoxidation rate. They found that the half-life for the conversion of oxymyoglobin to metmyoglobin was 3.3 days, 11 h, and less than 30 min at pH 9.1, 7.0, and 5.0, respectively. On the other hand, the capacity of meat to reverse the oxidation of metmyoglobin back to deoxymyoglobin (metmyoglobin reducing ability) is impaired by low pH (Zhu and Brewer, 1998; Bekhit and Faustman, 2005).

Denaturation of muscle proteins from abnormally low ultimate pH reduces the solubility of a number of proteins and their ability to bind water. As a result, large amounts of water migrate from inside the muscle fibers to the extracellular space, which increase light reflectance (Swatland, 2008). Additionally, low ultimate pH causes the loss of large amounts of water-soluble proteins including myoglobin due to the elevated loss of water through purge and drip. This results in a paler meat color. On the contrary, high ultimate pH causes meat color to appear darker due to greater water holding capacity and lower protein loss and denaturation. Consequently, light absorbance increases and lower reflectance of lights makes meat appear darker. Additionally, high ultimate pH promotes greater activity of oxygen-scavenging enzymes. This reduces oxygen available to bind myoglobin, resulting in more deoxymyoglobin formation rather than oxymyoglobin. (reviewed by King (née Turner) and Whyte, 2006). In totality, high ultimate pH reduces protein denaturation and improves myoglobin reducing ability and redox stability.

Water holding capacity

Many of the physical characteristics of meat such as tenderness, juiciness, firmness, and color improve as the content of water in the meat increases. Muscle tissue typically contains about 70-75% water. While, about 85% of this water is located within the myofiber, mainly in the space between thin and thick filaments, the remaining 15% is located in the extracellular space (Warriss, 2010). Muscle-based moisture can exist in bound, immobilized, and free forms. Bound water is water that is tightly and directly associated with muscle proteins through ion-dipole interactions (reviewed by Pearce et al., 2011). This water has extremely low mobility and is very resistant to external forces like freezing or heating. Yet, bound water represents only about 4-5% of total muscle water (Aberle et al., 2001). Immobilized water, also known as entrapped water, accounts for the largest portion (80-85%) of total water in the muscle and has a partially restricted mobility (Fennema, 1985; Pearce et al., 2011). Immobilized water is sequestered inside the muscle fiber by steric effects or by the formation of hydrogen bonding with muscle proteins and bound water (reviewed by Huff-Lonergan and Lonergan, 2005; reviewed by Pearce et al., 2011). During the conversion of muscle to meat, immobilized water is subject to migration into the free water compartment and can be subsequently lost as purge. Free water represents around 10% of muscle water and is held only by weak capillary forces in the interstitial space.

Water holding capacity (WHC) describes the ability of fresh meat to hold its water under external forces such as gravity, pressing, cutting, or heating (Aberle et al., 2001). WHC is an important quality attribute as it influences the yield and the quality of fresh and processed meat products. Many factors like pH, steric effect, and postmortem proteolysis influence WHC by altering the amount and location of moisture in muscle (Northcutt et al., 1994; reviewed by Huff-Lonergan and Lonergan, 2005; reviewed by Pearce et al., 2011). Among the three forms of water in the muscle, immobilized water is most affected by the biochemical changes occurring during the conversion of muscle to meat and, therefore, an important determinant of WHC (reviewed by

Huff-Lonergan and Lonergan, 2005). For meat processors, the more immobilized water preserved in the meat the greater the WHC.

During the process of converting muscle to meat, stored muscle glycogen is utilized in an attempt to replenish ATP levels through anaerobic metabolism. As a result of this catabolic pathway, lactate and hydrogen ions (H^+) accumulate in the muscle, causing the pH to gradually drop from 7.2 to an ultimate pH of around 5.5-5.7. Meat with low ultimate pH, close to the p*I* of major myofibrillar proteins has minimal WHC. Used in this case, p*I* is defined as the pH where the number of positive and negative charges on protein side groups is approximately equal. Consequently, these groups are attracted to each other, meaning the number of reactive groups available for water to react with is minimal (reviewed by Huff-Lonergan and Lonergan, 2005). Additionally, the attraction between protein reactive groups reduces the repulsive force between myofilament proteins. This causes shrinkage of the interfilament space and subsequently a reduction in the space available to hold water. The amount of space between the myofilaments is also influenced by the contractile state (sarcomere length) of the muscle. Formation of cross-bridges between the thick and thin filaments during the development of rigor mortis reduces the size of sarcomere, both longitudinally and laterally. Therefore, a reduction in the physical space for water to reside occurs with rigor development, which allows for more water loss through drip and purge (Offer et al., 1989). Rapid pH decline coupled with high muscle temperature early postmortem causes the denaturation of approximately 20% of muscle proteins, leading to the loss of functionality and the ability of protein to hold water (Honikel and Kim, 1986; reviewed by Huff-Lonergan and Lonergan, 2005).

Texture

Texture is usually described as the most important organoleptic attribute of meat because it affects the consumer's initial assessment and the overall acceptance of cooked meat. Texture is a complex concept that involves several attributes including, but not limited to tenderness,

juiciness, firmness, and cohesiveness. Among these textural properties, tenderness and juiciness are considered the most significant factors affecting the consumer satisfaction. Development of meat texture is a function of the interactions between the inherent traits of the animal, pre-slaughter extrinsic factors, and biochemical processes that occur during the conversion of muscle to meat. Postmortem factors that contribute to the texture of the meat include the rate and the extent of postmortem pH decline, carcass temperature, contractile state, proteolysis, and their interactions.

Postmortem metabolism is arrested in muscle as a result of glycogen and/or adenine nucleotides exhaustion or by pH inactivation of the glycolytic enzyme PFK-1 (Henckel et al., 2002; England et al., 2014, 2016). Regardless of the means, once ATP is depleted, actomyosin cross-bridges are formed, leading to the development of muscle stiffness. However, as postmortem proteolysis proceeds, muscle cytoskeletal proteins are degraded and meat becomes less stiff and more tender. It is well-documented that the degradation of the two cytoskeletal proteins titin and nebulin is associated with an increase in meat tenderness (Huff-Lonergan et al., 1995). Meat tenderness is biomodal and decreases as ultimate pH increases from 5.4 to 6.0, then improves as ultimate pH increases from 6.0 to 7.0 (Watanabe and Devine, 1996; Lomiwes et al., 2014). The reduction in tenderness around pH 6.0 is associated with an impaired degradation of titin and nebulin. This is thought to result from minimal activity of the two major proteolytic enzymes calpain and cathepsin (Watanabe and Devine, 1996; Lomiwes et al., 2014). Meat texture is also affected by the rate of pH decline. Rapid pH decline increases protein denaturation and water loss resulting in undesirable soft texture.

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Chapter 2 – Net lactate accumulation and low buffering capacity explain low ultimate pH in the *longissimus lumborum* of AMPKγ3^{R200Q} mutant pigs

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Author Contributions

Sulaiman K. Matarneh performed all experiments and helped write the article.

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Emily M. Oliver helped with experiments.

David E. Gerrard directed the research and helped write the article.

Abstract

Postmortem lactate accumulation in skeletal muscle is linearly associated with the extent of pH decline. Yet, pigs harboring the AMPKγ3^{R200Q} mutation produce meat with similar lactate levels to that of wild-type pigs but have a lower ultimate pH. We hypothesized that lower initial lactate levels and (or) lower buffering capacity in muscle of these pigs may help explain this discrepancy. *Longissimus lumborum* muscle samples were harvested at 0 and 1440 min postmortem from AMPKγ3^{R200Q} and wild-type pigs. As expected, AMPKγ3^{R200Q} muscle exhibited a lower ultimate pH but similar lactate levels to that of wild-type pigs at 1440 min postmortem. However, the total net lactate produced postmortem was greater in the AMPKγ3^{R200Q} muscle due to lower initial lactate levels at 0 min postmortem. Buffering capacity measured over the pH range of 5.5–7.0 was also lower in AMPKγ3^{R200Q} muscle. Greater net

lactate accumulation postmortem (i.e., glycolytic flux) coupled with a lower buffering capacity explains the lower ultimate pH of meat from AMPK$\gamma3^{R200Q}$ pigs.

Keywords: Acid meat; Lactate; Buffering capacity; Postmortem glycolysis.

Introduction

The extent of postmortem pH decline is widely valued as an indicator of fresh meat quality (Briskey, 1964; Monin & Sellier, 1985; reviewed by Van Laack, Kauffman, & Greaser, 2001). Generally, meat with an ultimate pH around 5.5–5.7 possesses the most desirable fresh quality characteristics (Mullen & Troy, 2005; reviewed by Van Laack et al., 2001), while meat with an abnormally low ultimate pH, closer to the isoelectric point of the myofibrillar proteins (pH 5.1–5.2), has a lower water holding capacity, paler color, reduced protein extractability and poor processing yield (Enfält, Lundström, Hansson, Johansen, & Nyström, 1997; Joo, Kauffman, Kim, & Park, 1999; Lundström, Andersson, & Hansson, 1996). To date, the exact biochemical mechanisms responsible for breaching the normal ultimate pH of meat remains unknown. While the frequency and predictability of this pork quality defect makes it difficult to study, the AMP-activated protein kinase $\gamma3^{R200Q}$ (AMPK$\gamma3^{R200Q}$) mutation found in some pigs reliably produces meat with a low ultimate pH and reduced pork quality characteristics.

The AMPK$\gamma3^{R200Q}$ or Rendement Napole (RN^-) mutation most notably found in Hampshire pigs is a single base substitution mutation in the PRKAG3 gene, encoding the $\gamma3$ regulatory subunit of AMPK. The mutation results from a single amino acid substitution of arginine to glutamine at residue 200 which results in a constitutively active AMPK (Milan et al., 2000). This muscle-specific mutation is restricted to glycolytic muscles, such as the *longissimus lumborum* muscle of pigs, and ironically increases the glycolytic and oxidative attributes of the

muscle simultaneously (Copenhafer, Richert, Schinckel, Grant, & Gerrard, 2006; Estrade, Ayoub, Talmant, & Monin, 1994; Scheffler et al., 2014). For reasons not yet known, muscle from these pigs reaches a low ultimate pH around 5.2 and as such, is sometimes referred to as acid meat (Milan et al., 2000).

Muscle acidification results from an accumulation of hydrogen ions (H^+) produced by ATP hydrolysis occurring in postmortem muscle as it attempts to remain within the homeostatic set-points. At the same time, however, the ability of the muscle to buffer against this charge build-up can also impact the ultimate pH of muscle during its conversion to meat (Bendall, 1973; Honikel & Hamm, 1974). Indeed, glycolytic flux under anaerobic conditions results in significant lactate and H^+ accumulation and as such, a negative linear relationship is often observed when muscle pH is plotted against lactate levels, especially if plotted over the entire postmortem period (Bendall, 1973). Based on this relationship and the fact that AMPK$\gamma3^{R200Q}$ mutant pigs deposit greater muscle glycogen, this lower ultimate pH is often attributed to an extended glycolysis postmortem. Yet, AMPK$\gamma3^{R200Q}$ muscle accumulates similar lactate levels at 1440 min postmortem suggesting glycolytic flux in dying muscle is similar across genotypes (Copenhafer et al., 2006; Enfält et al., 1997; Lundström, Enfält, Tornberg, & Agerhem, 1998; Monin & Sellier, 1985). To our knowledge, however, no studies have assessed the net accumulation in lactate (final lactate content − initial lactate content) produced or the buffering capacity in AMPK$\gamma3^{R200Q}$ mutant muscle postmortem. Therefore, the lack of a clear relationship between final lactate levels and ultimate pH in muscle postmortem may be due in part to different initial lactate concentrations and (or) buffering capacities in the muscle, especially in those pigs harboring the AMPK$\gamma3^{R200Q}$ genetic mutation.

Materials and methods

Genotyping

The polymerase chain reaction-restriction fragment length polymorphism (PCR-RLFP) technique was used to determine animal genotypes as described by (Meadus, MacInnis, Dugan, & Aalhus, 2002) with modifications. Briefly, DNA was extracted from tissue samples collected at birth. DNA was amplified using PCR and the resulting products were digested with the restriction enzyme BsrBI. Digested PCR products were separated on a 2% agarose gel, stained with ethidium bromide and visualized under UV light. Because AMPKγ3^{R200Q} is a dominant mutation, both homozygous (RN^{-}/RN^{-}) and heterozygotes (RN^{-}/rn^{+}) pigs at the RN locus were utilized.

Muscle sampling

A total of 14 market-weight pigs (100–125 kg, 7 AMPKγ3^{R200Q} mutant and 7 wild-type) were slaughtered in the Virginia Tech Meat Center using standard commercial procedures. Muscle samples were excised from the *longissimus lumborum* muscle at 0 min (within 5 min of exsanguination) and 1440 min postmortem. Samples were immediately frozen in liquid nitrogen, and stored at − 80 °C.

pH measurement

Muscle pH samples were prepared using the iodoacetate method as described by Bendall (1973) with a slight modification. Frozen muscle samples (~ 0.1 g) were lysed using a Tissue Lyser II system (Qiagen, Boston, MA, USA) in 0.8 ml of ice-cold buffer containing 5 mM sodium iodoacetate and 150 mM KCl (pH 7.0). Samples were centrifuged, equilibrated to 25 °C, and measured directly using an Orion Ross Ultra pH glass electrode (Thermo Scientific, Pittsburgh, PA, USA).

Metabolite analysis

Muscle glycogen content was determined using ~ 0.1 g samples, heated at 90 °C for 2 h in 1 ml of 1.25 M HCl. After centrifugation at 13,000 rpm for 5 min, the resulting supernatants were neutralized with 1.25 M KOH (Bergmeyer, 1984). For glucose 6-phosphate, glucose, lactate, adenine nucleotides (ATP, ADP, AMP), inosine monophosphate (IMP), and phosphocreatine (PCr) determination, samples (~ 0.1 g) were lysed in 1 ml of ice-cold 0.5 M perchloric acid. Homogenates were centrifuged at 13,000 rpm for 5 min. Supernatants were neutralized with 2 M KOH (Bergmeyer, 1984). Glycogen, glucose, glucose 6-phosphate, and lactate concentrations were measured using previous reported methods modified for a 96-well plate (Hammelman et al., 2003). All metabolites were analyzed at room temperature and measured spectrophotometrically at 340 nm. Adenine nucleotides and IMP contents were determined by high-performance liquid chromatography (HPLC) according to Bernocchi et al. (1994) and Williams, Vidt, and Rinehart (2008). Samples were filtered (PTFE 0.22 μm pore size) then injected into an HP Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA). Separation was achieved using a C18 2.6 μm 50 × 4.6 mm column (Thermo Scientific, Pittsburgh, PA, USA). Two mobile phases were used to enable gradient elution: mobile phases A contained 100 mM KH_2PO_4, 5 mM tetrabutylammonium hydrogen sulfate (TBAHS), and 2.5% (vol/vol) acetonitrile, pH 6.0 and mobile phase B contained 100 mM KH_2PO_4, 5 mM TBAHS, and 25% (vol/vol) acetonitrile, pH 5.5. Flow rates were maintained at 1.25 ml/min. Detections were performed at room temperature at a wavelength of 260 nm. Phosphocreatine concentrations were measured by HPLC using isocratic elution. The HPLC conditions were the same as described above, except that only mobile phase A was used and the UV detector was set at 205 nm.

Buffering capacity

Muscle buffering capacity was determined as described previously (Henckel, Jorgensen, & Jensen, 1992; Puolanne & Kivikari, 2000). Briefly, ~ 1 g of the 1440 min meat samples was homogenized at 1:10 (wt/vol) in 5 mM sodium iodoacetate and 150 mM KCl (pH 7.0). Homogenates were equilibrated to 25 °C and titrated using 0.1 M NaOH. Sample pH was measured using an Orion Ross Ultra pH glass electrode (Thermo Scientific, Pittsburgh, PA, USA). Buffering capacity (BC) was calculated using the formula BC = ΔB / ΔpH, where, ΔB is the increment of base expressed as μmol NaOH/g of tissue and ΔpH is the corresponding change in pH. The titration curve for each animal was obtained by plotting BC values against the average of the two respective pH values.

Statistical analysis

The data were analyzed using the mixed model of JMP (SAS institute Inc., Cary, NC). The statistical model included the fixed effect of genotype and the random effect of animal. The related Fisher's LSD was calculated and differences were considered significant at $P \leq 0.05$. All data are presented as least-squares (LS) means ± SE.

Results

The extent of postmortem pH decline was affected by genotype (Fig. 2-1). No difference in pH at 0 min was observed between AMPKγ3^{R200Q} and wild-type genotypes. However, lower ($P = 0.015$) pH at 1440 min was detected in the mutant pigs.

As expected, the carriers of the AMPKγ3^{R200Q} mutation had more than twice the amount of muscle glycogen at 0 min postmortem ($P = 0.0001$; Fig. 2-2A) compared to the wild-type animals. At 1440, the mutant pigs maintained their greater glycogen levels compared to the wild-types ($P < 0.0001$; Fig. 2-2A). No difference in glucose 6-phosphate concentration among genotypes was observed at 0 min, however, AMPKγ3^{R200Q} muscle possessed greater glucose 6-

phosphate concentration at 1440 min postmortem ($P = 0.02$; Fig. 2-2B). Although the difference was relatively small, lower muscle glucose was detected in the mutant pigs at 0 min postmortem ($P = 0.01$), but AMPKγ3^{R200Q} muscle contained greater ($P = 0.005$) glucose at 1440 min (Fig. 2-2C). Collectively, total glycogen degradation (ΔG6P + Δglucose + (Δlactate/2)) from 0 to 1440 min postmortem was greater in the mutant pigs compared to that of wild-type pigs ($P = 0.0008$; Fig. 2-2D).

Lactate concentrations were not different ($P = 0.87$) among genotypes at 1440 min (Fig. 2-3). Despite the lack of difference in lactate concentration at 1440 min, AMPKγ3^{R200Q} mutants exhibited lower ($P = 0.049$) initial lactate content at 0 min postmortem. Therefore, due to this initial difference, the AMPKγ3^{R200Q} pigs produced significantly greater ($P = 0.046$) lactate postmortem than wild-type pigs. Mean Δlactate values were 59.9 ± 2.65 and 71.0 ± 2.65 for wild-type and AMPKγ3^{R200Q} pigs, respectively.

A weak but significant ($R^2 = 0.35$, $P = 0.03$; Fig. 2-4A) relationship between ultimate pH and lactate at 1440 min was observed when data from both genotypes were combined. However, lactate at 1440 min was not related ($R^2 = 0.11$, $P = 0.25$; Fig. 2-4B) to ΔpH. Strong relationships ($R^2 \geq 0.59$, $P = 0.001$) were observed between ultimate pH and ΔpH in relation to Δlactate (Fig. 2-4C and D, respectively).

Phosphocreatine content in AMPKγ3^{R200Q} pigs was numerically greater but not statistically different at 0 min. At 1440 min, PCr contents were below detection in both genotypes (Table 2-1). A strong tendency for greater ($P = 0.06$) ATP in the mutant pigs was detected at 0 min while ATP was below detection at 1440. AMPKγ3^{R200Q} muscle exhibited lower ADP contents than wild-types at 0 and 1440 min postmortem ($P \leq 0.001$). No differences in AMP levels were observed among genotypes at either 0 or 1440 min postmortem. While lower

($P = 0.003$) IMP concentration was detected in the AMPKγ3^{R200Q} mutant pigs at 0 min, no difference was observed at 1440 min postmortem (Table 2-1).

The titration curves of *longissimus lumborum* muscle of wild-type and AMPKγ3^{R200Q} pigs over the pH range 5.5–7 are shown in Fig. 2-5. The two regression lines were almost coincident each other between pH 7–6. However, below pH of 6, the slope of the regression line for AMPKγ3^{R200Q} pigs remains strong, indicating a lower buffering capacity in the mutant pigs. Minimal buffering capacity was observed around pH 5.6 for both genotypes. From the titration curve of each animal we then estimated the buffering capacity at pH 5.5, 6, and a cumulative buffering capacity of the pH range 7–5.5 (Fig. 2-6). While no difference in buffering capacity was observed between the two genotypes at pH 6, lower buffering capacity at pH 5.5 ($P = 0.008$) and over the pH range 7–5.5 ($P = 0.002$) were observed in the AMPKγ3^{R200Q} pigs.

Discussion

Defining the exact mechanisms responsible for variations in the ultimate pH of meat has proven challenging. Even so, using the AMPKγ3^{R200Q} pigs as a model for exaggerated pH decline, while likely unique to this mutation, has provided much insight into those factors affecting postmortem metabolism (England, Matarneh, Scheffler, Wachet, & Gerrard, 2015; Scheffler, Kasten, England, Scheffler, & Gerrard, 2014; Scheffler, Scheffler, Kasten, Sosnicki, & Gerrard, 2013). Extended glycolysis was originally thought by many as the primary culprit responsible for lowered ultimate pH, primarily based on the existence of the AMPKγ3^{R200Q} pig and its inherent muscle and meat characteristics, high glycogen and acid meat, respectively. However, similar muscle lactate values at 1440 min postmortem argue against this construct (Copenhafer et al., 2006; Enfält et al., 1997; Lundström et al., 1998; Monin & Sellier, 1985). Results of this study nonetheless suggest that net lactate accumulation coupled with a lower buffering capacity may

be responsible for the lowered ultimate pH of meat observed in AMPKγ3^{R200Q} pig muscle, rather than a function of elevated substrate level.

Muscle from AMPKγ3^{R200Q} pigs is somewhat contradictory in terms of energy metabolism. Activated AMPK increases energy generating pathways within the cell and blunts those pathways or mechanisms that utilize energy (reviewed by Towler & Hardie, 2007). Activated AMPK also activates the transcriptional co-activator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), which ultimately leads to mitochondrial biosynthesis (reviewed by Canto & Auwerx, 2009). The *longissimus lumborum* from AMPKγ3^{R200Q} mutant pigs contain greater oxidative capacity, as defined by greater mitochondrial protein content and enzyme activity, greater mitochondrial DNA, and higher mitochondrial oxygen consumption rate compared to non-mutant pigs (Estrade et al., 1994; Lebret et al., 1999; Scheffler, Kasten, England, Scheffler, & Gerrard, 2014; Scheffler et al., 2014). Moreover, this muscle inherently possesses lower lactate dehydrogenase and lower AMP deaminase activity (England et al., 2015; Granlund, Jensen-Waern, & Essén-Gustavsson, 2011; Lebret et al., 1999). While the aforementioned contributes directly to energy metabolism, an increase in the composition IIa and IIx muscle fiber types and their respective heavy chain gene expressions have been noted in AMPKγ3^{R200Q} pig muscle with a corresponding decrease in the faster contracting fibers compared to wild-type pig muscle (Lebret et al., 1999; Park, Gunawan, Scheffler, Grant, & Gerrard, 2009). Oxidative muscles typically exhibit a higher ultimate pH compared to those more glycolytic muscles (Wittmann, Ecolan, Levasseur, & Fernandez, 1994), yet muscle of these animals produces meat with a lower ultimate pH (Fig. 2-1). To achieve a low ultimate pH, muscle tissue must contain sufficient glycogen to provide carbohydrate for glycolytic flux. Indeed, the *longissimus lumborum* from AMPKγ3^{R200Q} pigs contains

approximately 100% more glycogen than wild-type pigs (Fig. 2-2A) and this level is in agreement with those reported previously (Copenhafer et al., 2006; Monin, Brard, Vernin, & Naveau, 1992). Moreover, AMPK$\gamma3^{R200Q}$ muscle contains increased glycogen phosphorylase and hexokinase activity (Granlund et al., 2011), which indicates a greater capacity to mobilize substrate (glucose 6-phosphate) especially under anaerobic conditions. The AMPK$\gamma3^{R200Q}$ pig is a unique mixture of oxidative and glycolytic characteristics and suggests that control of postmortem metabolism in the muscle may be likewise unique but nonetheless useful for studying the transformation of muscle to meat. Regardless, these data show the *longissimus lumborum* from AMPK$\gamma3^{R200Q}$ pigs is more oxidative in nature and suggests that lowered initial lactate concentrations may be a function of the greater mitochondria in muscle of these pigs prior to slaughter.

Congruent with these facts is that muscle of AMPK$\gamma3^{R200Q}$ pigs has greater initial PCr levels compared to that of their wild-type counter parts (Copenhafer et al., 2006). As muscle attempts to maintain cellular ATP levels postmortem, PCr is the initial reaction of the phosphogen system which rapidly regenerates ATP. After the bulk of PCr is utilized and ADP rises, muscle glycogen is broken down and metabolized to rephosphorylate ADP (Bendall, 1973; reviewed by Scheffler & Gerrard, 2007). While no detectable differences were noted in PCr in the present study (Table 2-1), we have observed an increase previously (Copenhafer et al., 2006). Reasons for our failure to detect a difference is not readily known but could be related to differences in sampling postmortem as PCr disappearance is quite fast in muscle. Either way, given the elevated energy status of the tissue resulting from this mutation, a likely scenario is that AMPK$\gamma3^{R200Q}$ pigs rely more heavily on PCr as a means of ATP regeneration rather than glycolysis early postmortem. This notion is supported by lower glucose, ADP, IMP, and a

tendency toward greater amounts of ATP in the mutant pig muscle (Fig. 2-2 and Table 2-1). Alternatively, the increased mitochondrial capacity of the AMPK$\gamma 3^{R200Q}$ muscle is capable of maintaining ATP levels prior to PCr catabolism.

Even though we observed a significantly lower initial lactate levels in the mutant pig muscle at 0 min postmortem (Fig. 2-3), a corresponding change in muscle pH was not observed between genotypes at this time (Fig. 2-1). Muscle lactate accumulation depends on the competition between the cytosolic enzyme LDH and the mitochondria for the pyruvate derived from glycolysis. Under aerobic conditions, pyruvate is rapidly shuttled into the mitochondria and converted to acetyl CoA and used in the citric acid cycle to produce ATP through oxidative phosphorylation and the electron transport chain (Berg, Tymoczko, & Stryer, 2007). In living tissue, lactate and H^+ produced during anaerobic metabolism is removed from muscle tissues via a lactate/H^+ transporter. In addition, H^+ can also be removed by Na^+/H^+ exchanger as well as bicarbonate-dependent systems (reviewed by Juel, 1996). Therefore, the rate of lactate removal from muscle tissue is lower than the rate of H^+ removal, which might explain the lack of difference in pH associated with disparity in lactate between genotypes at 0 min postmortem. Finally, we have recently shown that functioning mitochondria exist postmortem (Scheffler, Matarneh, England, & Gerrard, 2015) and as eluded to earlier, they may function to lower lactate levels by lowering the availability of pyruvate in the muscle especially early postmortem before anaerobic conditions prevail.

Previously, our laboratory established that postmortem glycolysis stops because phosphofructokinase-1 (PFK-1) becomes pH inactivated beginning at pH 5.9 (England, Matarneh, Scheffler, Wachet, & Gerrard, 2014). We have also demonstrated, however that

glycolytic intermediates traversing PFK-1 are still ultimately metabolized to lactate until a pH of 5.1 is reached in the tissues. This is particularly germane for red and white muscles differing in their ultimate pH because the rate of glycolytic flux differs substantially with muscle type (unpublished data). Given the rate of pH decline in muscle from AMPK$\gamma3^{R200Q}$ pigs is similar to that occurring in wild-type pig muscle suggests the rate of glycolysis is not different between AMPK$\gamma3^{R200Q}$ and wild-type muscles. If this is indeed the case, then the exact mechanism by which these deviates are capable of breaching the normal range of muscle pH values 1440 min postmortem remains obscure. However, for the AMPK$\gamma3^{R200Q}$ pigs to produce enough H^+ to reach an ultimate pH near 5.2 or 5.3, glycolysis must function longer or more substrates must be capable of traversing the PFK-1 reaction sooner. While we have no evidence the latter is occurring, we have shown that AMP deaminase activity is lower in AMPK$\gamma3^{R200Q}$ muscle arguing that AMP levels are maintained at later times postmortem and may contribute to a protracted period of glycolysis and lower ultimate pH (England et al., 2015).

Our current findings indicate that net lactate accumulation ($R^2 = 0.59$; Fig. 2-4C) is more useful in predicting ultimate pH than total lactate ($R^2 = 0.35$; Fig. 2-4A) in both AMPK$\gamma3^{R200Q}$ and wild-type pig muscle. This conclusion may help explain some of the variation observed in meat quality development with changes in animal behavior, antemortem handling, and fasting. There is little question that these activities impact meat quality (reviewed by England, Scheffler, Kasten, Matarneh, & Gerrard, 2013). Specifically, Brown, Warriss, Nute, Edwards, and Knowles (1998) showed muscle lactate parallels handling protocols pre-slaughter. Based on our data, we propose that differences in pre-slaughter stress and lactate clearance from the tissues, either through mitochondrial function or transport of H^+ directly or indirectly to the circulation may in

part explain the lackluster relationships observed between the ultimate pH of muscle and final lactate concentrations.

Decline in muscle pH postmortem is the net result of H^+ produced by glycolysis minus those bound and neutralized by negative charged entities throughout the cell. Without this buffering capacity, muscle pH would drop precipitously as a result of H^+ accumulation during a short bout of exercise or in this case, postmortem metabolism (reviewed by Pösö & Puolanne, 2005). The minimum (at pH ~ 5.6) and maximum (at pH ~ 6.7) buffering capacity values (Fig. 2-5) obtained in the present study were in agreement with those previously obtained in porcine *longissimus* muscle (Puolanne & Kivikari, 2000). Van Laack et al. (2001) first suggested that buffering capacity may explain differences in ultimate pH between muscles with similar lactate levels. Buffering capacity is greater in white muscles than in those redder, more oxidative muscles (Kyla-Puhju, Ruusunen, Kivikari, & Puolanne, 2004; Puolanne & Kivikari, 2000; Rao & Gault, 1989). While non-dipeptide compounds are fairly constant between muscles with different fiber type composition (Sewell, Harris, Marlin, & Dunnett, 1992) and also between different species (Harris, Marlin, Dunnett, Snow, & Hultman, 1990), Rao and Gault (1989) explained differences in buffering capacity between white and red muscle fibers by differences in the contents of inorganic phosphate and carnosine. We measured total acid extractible phosphates (glucose 6-phosphate, fructose 6-phosphate and inorganic phosphate) and carnosine levels for both genotypes, but no differences were detected (data not shown). Though, the difference in cumulative buffering capacity in the current study was quite small (2.3 μmol $H^+ \cdot pH^{-1} \cdot g^{-1}$) and may be the reason why we were unable to detect a difference in the content of total phosphate and carnosine.

The cumulative buffering capacity in the AMPKγ3^{R200Q} pigs obtained in this study essentially means that for every 50 μmol H^+/g produced, muscle pH declines 1.0 unit. In this study, AMPKγ3^{R200Q} pigs produced approximately 10 μmol/g more lactate than the wild-type pigs. Because one H^+ molecule is produced for every lactate molecule (reviewed by Hamm, 1977), a simple substitution calculation argues the extra lactate produced by the mutant pigs is sufficient to reduce the pH of the muscle by 0.2 pH units. In this study, differences in pH between genotypes were about 0.2, meaning that the difference in pH can be explained by the greater glycolytic flux alone. While greater glycolytic flux explains the differences in ultimate pH, between AMPKγ3^{R200Q} and normal pig muscle, differences in buffering capacity may be partially to blame especially given muscle in these pigs is more oxidative, which by nature has a lower buffering capacity.

Conclusions and implications

Results presented herein show that the *longissimus lumborum* muscle of AMPKγ3^{R200Q} pigs has greater glycolytic flux, albeit with similar final lactate concentrations equal to that of wild-type pig muscle. AMPKγ3^{R200Q} pigs exhibited greater postmortem glycogen degradation, net lactate production, and lower ultimate pH compared to wild-type pigs. Furthermore, a lower buffering capacity below pH 6 was observed in the mutant pigs, which may further contribute to the lower ultimate pH. However, the mechanism responsible for the greater glycolytic flux in the AMPKγ3^{R200Q} mutant pigs remains to be explained. Even so, however, these findings expand our knowledge of understanding better of how ultimate pH is determined. To that end, we have updated our working model (England et al., 2014, 2015) of those factors controlling the rate and the extent of pH decline during the conversion of muscle to meat (Fig. 2-7).

Acknowledgments

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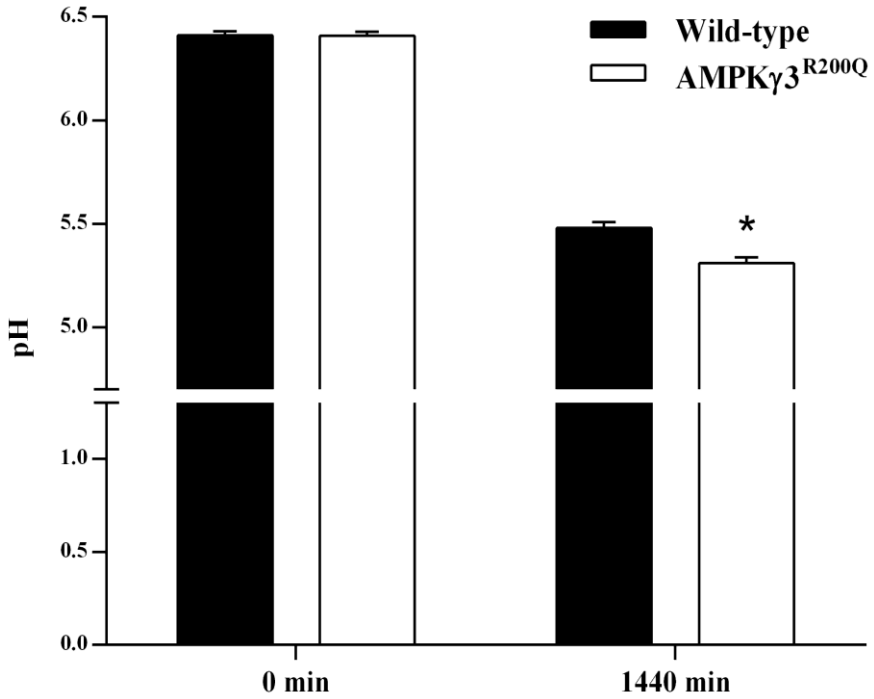

Figure 2-1. The pH of *longissimus lumborum* muscle at 0 and 1440 min postmortem for wild-type and AMPKγ3^{R200Q} genotypes. Data are LS means ± SE. *indicates significant difference within a time point ($P < 0.05$).

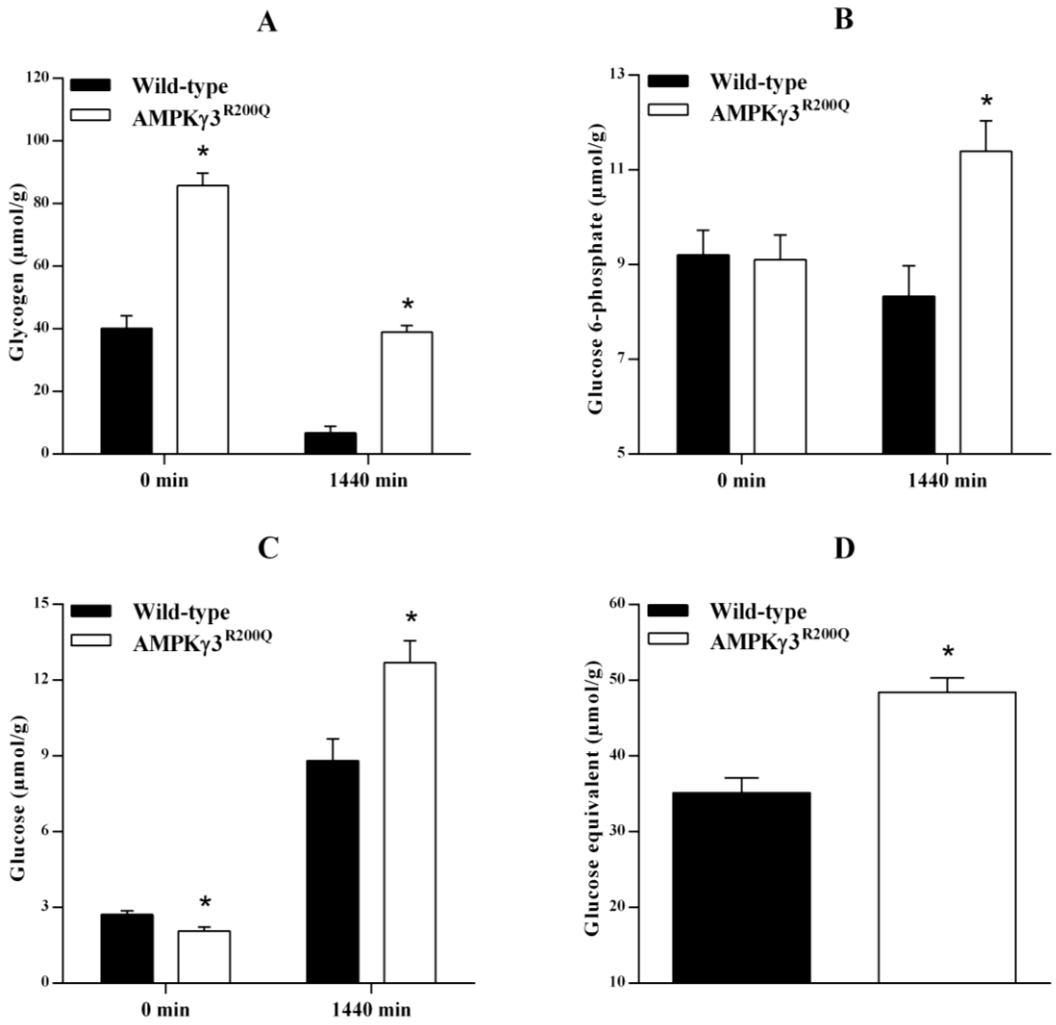

Figure 2-2. Metabolite contents and total glycogen degradation in *longissimus lumborum* muscle of wild-type and AMPKγ3^{R200Q} genotypes. Glycogen (µmol/g) at 0 and 1440 min postmortem (A). Glucose 6-phosphate (µmol/g) at 0 and 1440 min postmortem (B). Glucose (µmol/g) at 0 and 1440 min postmortem (C). Total postmortem glycogen degradation (glucose equivalent, µmol/g) (D). Data are LS means ± SE. *Indicates significant difference within a time point for A, B, and C and between genotypes for D ($P < 0.05$).

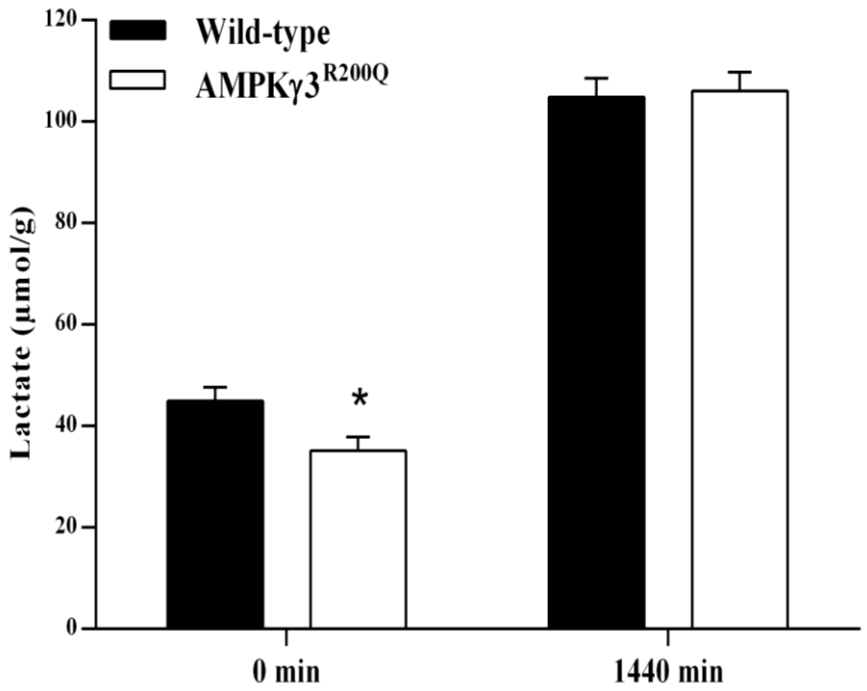

Figure 2-3. Lactate at 0 and 1440 min postmortem (µmol/g) in *longissimus lumborum* muscle of wild-type and AMPKγ3^{R200Q} genotypes. Data are LS means ± SE. *Indicates significant difference within a time point ($P < 0.05$).

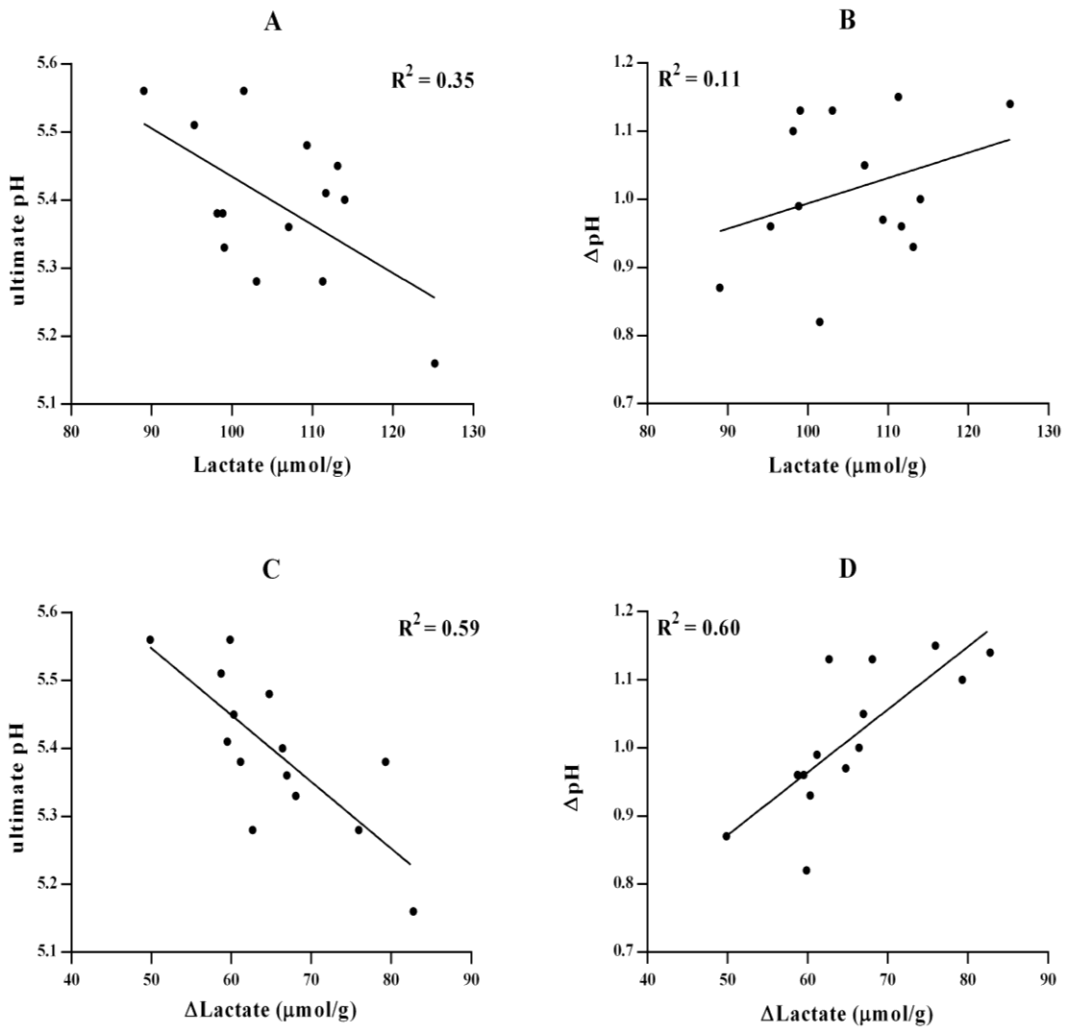

Figure 2-4. Relationship between ultimate pH and lactate (µmol/g) at 1440 min postmortem (A), ΔpH and lactate (µmol/g) at 1440 min postmortem (B), ultimate pH and Δlactate (C), and ΔpH and Δlactate (µmol/g) (D) in *longissimus lumborum* muscle regardless genotype.

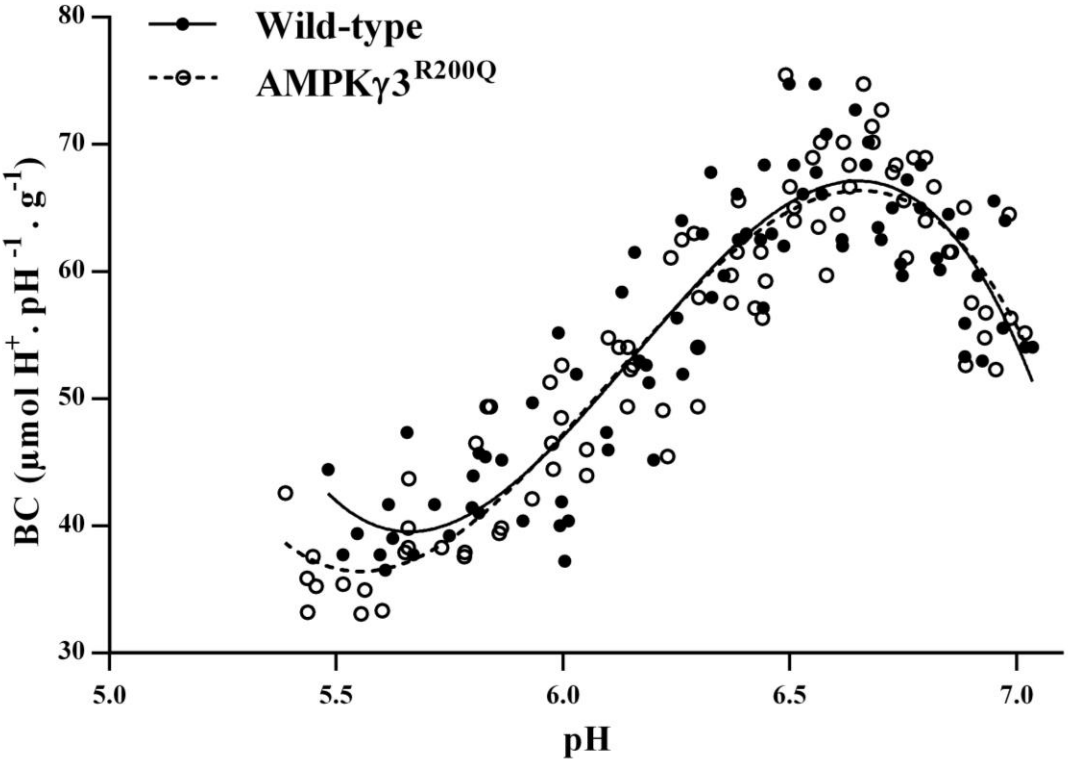

Figure 2-5. Titration with NaOH of *longissimus lumborum* muscle homogenates (1:10 wt/vol) of wild-type and AMPKγ3^{R200Q} genotypes over the pH range 5.5–7. The solid line represents wild-type and the broken line represents AMPKγ3^{R200Q}.

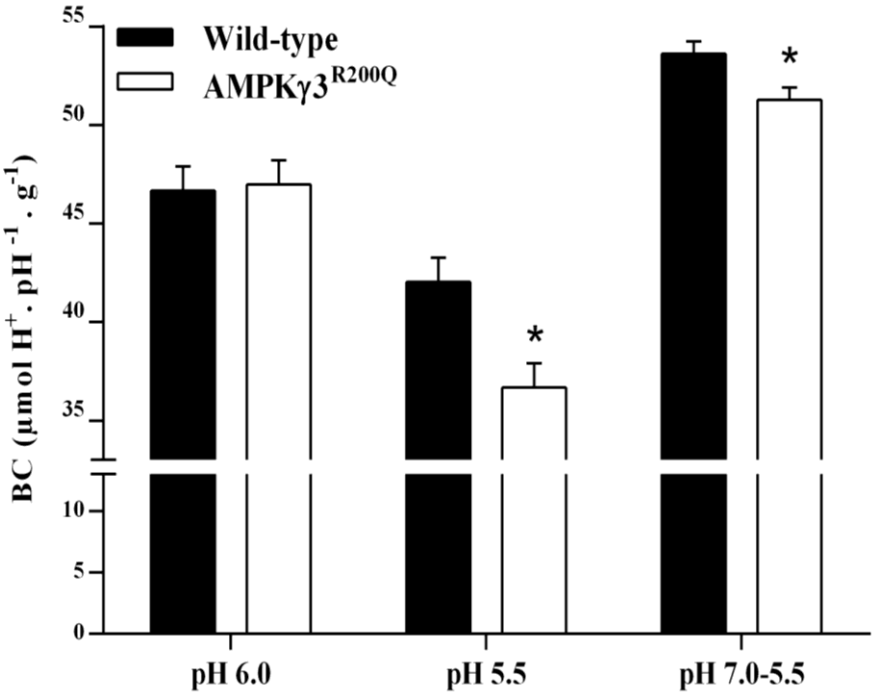

Figure 2-6. Buffering capacity (μmol H^{+}·pH^{-1}·g^{-1}) at pH 5.5, 6.0, and over the pH range 5.5–7.0 in *longissimus lumborum* muscle of wild-type and AMPKγ3^{R200Q} genotypes. Data are LS means ± SE. *Indicates significant difference within each pH ($P < 0.05$).

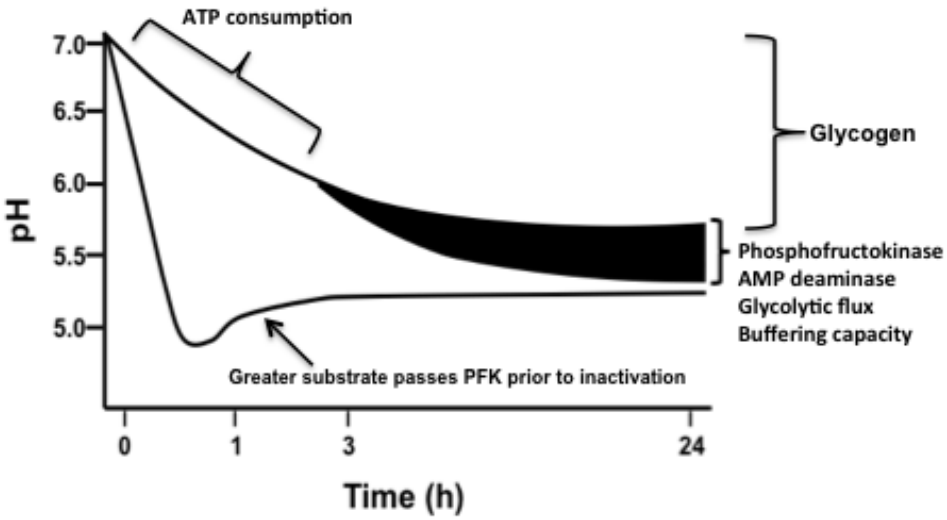

Figure 2-7. Working model of the factor controlling the rate and extent of postmortem metabolism.

Table 2-1. Phosphocreatine (PCr), adenine nucleotides (ATP, ADP, AMP), and inosine monophosphate (IMP) (µmol/g) in *longissimus lumborum* muscle of wild-type and AMPKγ3^{R200Q} pigs. Data are LS means ± pooled SE. [a,b]Means lacking a common superscript differ within a time point ($P < 0.05$). BD = Below limit of detection.

	Metabolite				
Item	PCr (µmol/g)	ATP (µmol/g)	ADP (µmol/g)	AMP (µmol/g)	IMP (µmol/g)
0 min					
Wild-type	8.07 ± 2.5	5.30 ± 0.18	1.04 ± 0.03[a]	0.16 ± 0.03	1.10 ± 0.16[a]
AMPKγ3^{R200Q}	11.14 ± 2.0	5.67 ± 0.18	0.77 ± 0.03[b]	0.10 ± 0.03	0.47 ± 0.16[b]
P-value	0.40	0.06	0.001	0.18	0.003
1440 min					
Wild-type	BD	BD	0.23 ± 0.01$_a$	0.20 ± 0.01	6.95 ± 0.28
AMPKγ3^{R200Q}	BD	BD	0.19 ± 0.01[b]	0.21 ± 0.01	6.58 ± 0.28
P-value	-	-	0.0002	0.59	0.32

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Chapter 3 – A mitochondrial protein increases glycolytic flux

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Author Contributions

Sulaiman K. Matarneh performed all experiments and helped write the article.

Eric M. England helped with experiments.

Tracy L. Scheffler helped with experiments.

Con-Ning Yen helped with experiments.

Jordan C. Wicks helped with experiments.

David E. Gerrard directed the research and helped write the article.

Abstract

The purpose of this study was to determine the role of mitochondria in postmortem muscle metabolism. Isolated mitochondria were incorporated into a reaction buffer that mimics postmortem glycolysis with or without mitochondrial electron transport chain inhibitors. Addition of mitochondria lowered pH values at 240 and 1440 min regardless of inhibitors. Reduction in pH was accompanied by enhanced glycogen degradation and lactate accumulation. To explore the mechanism responsible for this exaggerated metabolism, mitochondrial preparations were physically disrupted and centrifuged. Resulting supernatants and pellets each were added to the *in vitro* model. Mitochondrial supernatants produced similar effects as those including intact mitochondria. To narrow further our target of investigation, mitochondrial supernatants were deproteinized with perchloric acid. The effect of mitochondrial supernatant

was lost after perchloric acid treatment. These data indicate that a mitochondrial-based protein is capable of increasing glycolytic flux in an *in vitro* model and may partially explain acid meat development in highly oxidative AMPK$\gamma 3^{R200Q}$ mutated pigs.

Key words: Mitochondria; Postmortem metabolism; pH; Glycolytic flux.

Introduction

During the process of converting muscle to meat, hydrogen ions (H^+) accumulate in the muscle and gradually lower the pH from 7.2 to an ultimate pH around 5.5. The rate and extent of postmortem pH decline can profoundly affect the quality characteristics of fresh meat. While the rate of pH decline is a function of muscle ATPase activity (Scopes, 1974), the exact biochemical mechanisms responsible for determining the ultimate pH of meat remain imprecise. Although great efforts have been made to determine factors influencing the extent of pH decline (England, Matarneh, Scheffler, Wachet, & Gerrard, 2014; Matarneh, England, Scheffler, Oliver, & Gerrard, 2015; Scopes, 1974; Van Laack, Yang, & Spencer, 2001), gaps in our knowledge still exist. This is partly a result of the ambiguity and over-simplification of events controlling postmortem metabolism.

Postmortem metabolism is usually viewed as exclusively anaerobic process, with glycogen being converted to lactate and H^+ that accumulate in the muscle and cause a drop in pH. However, attempts to predict accurately the extent of pH decline using only anaerobic metabolism have proven unreliable (Monin & Sellier, 1985; Scheffler, Scheffler, Kasten, Sosnicki, & Gerrard, 2013), suggesting other mechanisms may be involved in determining the extent of postmortem metabolism. Because mitochondrial respiration is impeded by the lack of oxygen, mitochondria are often considered irrelevant to postmortem metabolism. Yet,

mitochondria preserve functionality and structural integrity for several hours postmortem (Tang et al., 2005), and therefore represent a potential mechanism for altering postmortem metabolism. Indeed, we have recently shown that inclusion of functioning mitochondria to an *in vitro* model designed to mimics postmortem metabolism reduced the rate of ATP hydrolysis (Scheffler, Matarneh, England, & Gerrard, 2015), which strongly argues mitochondria impact postmortem metabolism. Further, mitochondria may alter cytosolic redox state (NAD^+/NADH ratio), which could impact glycolysis (Jong & Davis, 1983).

Our interest in the possible role of mitochondria in postmortem metabolism has mostly risen from studying pork quality in pigs harboring the AMPK$\gamma3^{R200Q}$ (RN^-) mutation. The postmortem pH decline in muscle from pigs carrying the AMPK$\gamma3^{R200Q}$ mutation is usually characterized by a normal rate initially, but continues to drop abnormally to an ultimate pH around 5.3 (Milan et al., 2000). The lower ultimate pH of AMPKγ_3^{R200Q} pigs is often attributed to a greater than normal glycolytic potential in resting muscle of these pigs compared to wild-types (Monin & Sellier, 1985). Yet, we have recently proposed that greater flux through glycolysis in muscle from AMPK$\gamma3^{R200Q}$ pigs causes a lower ultimate pH rather than greater tissue glycogen deposition (Matarneh et al., 2015). On the other hand, however, these pigs contain more mitochondria and retain greater oxidative capacity (Estrade, Ayoub, Talmant, & Monin, 1994; Scheffler et al., 2014), suggesting mitochondria may contribute in some way to the lower ultimate pH of muscle in these mutant pigs. Therefore, the purpose of this research was to examine the role of mitochondria in postmortem metabolism, specifically as related to acid meat development. However, testing the role of mitochondria *in vivo* is difficult. This is particularly onerous because modifying mitochondrial content of a muscle can alter confounding factors (i.e., glycolytic capacity) that can also impact postmortem pH decline (Petersen, Henckel, Maribo,

Oksbjerg, & Sørensen, 1997). To eliminate the potentially confounding effect, we used an *in vitro* system designed to recapitulate postmortem glycolysis (England et al., 2014; Scopes, 1973) and added 0.5 mg/ml isolated mitochondria with or without mitochondrial electron transport chain (ETC) inhibitors.

Materials and methods

Muscle sampling

All *pigs* used for this study were raised under the same feeding and management conditions at the Virginia Tech Swine Center *t*o approximately 115 kg live weight. Six crossbred (Yorkshire × Duroc; 3 males and 3 females) pigs were transported to the Virginia Tech Meat Science Center and harvested using standard commercial procedures. Muscle samples were excised from the *longissimus lumborum* muscle within 5 min of exsanguination. Samples were used for mitochondrial extraction or immediately snap frozen in liquid nitrogen, and stored at − 80 °C.

Mitochondria isolation and characterization

Mitochondria were isolated using the differential centrifugation method as described by Scheffler et al. (2014) with minor modification. Briefly, muscle samples were finely minced at 1:5 (wt/vol) in ice-cold isolation buffer (100 mM sucrose, 180 mM KCl, 50 mM Tris, 5 mM $MgCl_2$, 10 mM EDTA, 1 mM K-ATP, pH 7.4). Protease (subtilisin A) was added to the tissue suspension at 0.4 mg/ml before homogenization with a Potter-Elvehjem type homogenizer system (Glas-Col, Terre Haute, IN. USA). Homogenates were diluted with isolation buffer to achieve ~ 20 ml/g of tissue followed by filtering through cheese-cloth. Homogenates were then centrifuged at 1,000 × g for 10 min at 4 °C. Resulting supernatants were filtered again through cheese-cloth followed by centrifugation at 8,000 × g for 10 min at 4 °C. Mitochondrial pellets

were re-suspended by repeated pipetting in mitochondrial suspension buffer (220 mM mannitol, 70 mM sucrose, 10 mM Tris–HCl, and 1 mM EGTA, pH 7.4). Mitochondrial protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL. USA).

Intactness of mitochondria was evaluated by measuring citrate synthase activity in mitochondria preparations and the same preparations subjected to sonication for 2 min. Briefly, mitochondria were added to a buffer containing 85 mM Tris (pH 8.3), 0.5 mM oxaloacetate, 0.45 mM acetyl-CoA, and 0.1 mM dithionitrobenzoic acid. The assay was carried out at 37 °C and the increase in absorbance due to the formation of thionitrobenzoic acid was measured spectrophotometrically at 412 nm. Because inner mitochondrial membrane is impermeable to the substrates, the activity in the sonicated preparations (ruptured mitochondria) represents total citrate synthase activity as substrates are readily available to the enzyme. Percentage of intact mitochondria was calculated by comparing citrate synthase activity of the non-sonicated mitochondrial samples to that of the sonicated samples (Scheffler et al., 2015).

In vitro glycolysis model and experimental design

Frozen muscle samples were pulverized under liquid nitrogen and homogenized at 1:10 (wt/vol) in reaction buffer containing 40 mM glycogen, 60 mM KCl, 5 mM $MgCl_2$, 10 mM Na_2HPO_4, 30 mM creatine, 25 mM carnosine, 10 mM sodium acetate, 5 mM ATP, 0.5 mM ADP, and 0.5 mM NAD^+ (pH 7.4) (England et al., 2014). Either 0 or 0.5 mg/ml isolated mitochondria were incorporated into the *in vitro* model with or without a mitochondrial inhibitor cocktail (20 μM rotenone, 10 mM potassium cyanide, and 10 μM oligomycin, to inhibit mitochondrial complexes I, IV, and V, respectively). Aliquots were removed at 0, 30, 120, 240, and 1440 min for pH and metabolite analysis. Reaction vessels were maintained at 25 °C for the duration of the trial.

Longissimus lumborum muscles from six different pigs were used for mitochondrial isolation, while frozen portions were powdered and split between treatments (n = 6 per treatment).

In the second experiment, mitochondrial samples were homogenized with a Polytron homogenizer (Polytron PT-MR 2100, Kinematica AG, Switzerland) then centrifuged at 13,000 rpm for 5 min at 4 °C. The resulting supernatants were transferred to new tubes while pellets were re-suspended again in the mitochondrial suspension buffer. Mitochondrial supernatant and pellet were then tested in the *in vitro* model.

In the third experiment, mitochondrial supernatants were mixed with equal volume of 1 M perchloric acid (PCA) and incubated on ice for 15 min. After centrifugation at 13,000 rpm for 5 min at 4 °C, resulting supernatants were transferred to new tubes and the pH was adjusted to 7.4. Mitochondrial and PCA-treated supernatants were tested in the *in vitro* model. Number of experimental units per treatment, time of aliquots removal and conditions in the second and third experiments were consistent with the first experiment.

pH measurement

pH samples were prepared using the iodoacetate method as described by Bendall (1973). Briefly, four volumes of homogenate were removed from the reaction vessel and placed in a new tube containing one volume of 25 mM sodium iodoacetate and 750 mM KCl (pH 7.0). Samples were centrifuged at 13,000 rpm for 5 min at room temperature, equilibrated to 25 °C and measured directly using an Orion Ross Ultra pH glass electrode (Thermo Scientific, Pittsburgh, PA, USA).

Metabolite analysis

Aliquots for glycogen analysis were mixed at 1:1 ratio with 2.5 M HCl, heated at 90 °C for 2 h, centrifuged at 13,000 rpm for 5 min, and the resulting supernatant was neutralized with 1.25 M KOH (Bergmeyer, 1984). Aliquots for glucose, glucose 6-phosphate (G6P), lactate, adenine

nucleotides (ATP, ADP, AMP), and inosine monophosphate (IMP) were incubated for 20 min on ice with an equal volume of 1 M PCA. Following, samples were centrifuged at 13,000 rpm for 5 min, and the resulting supernatants were transferred to new tubes and neutralized with 2 M KOH (Bergmeyer, 1984). Glycogen, glucose, G6P, and lactate concentrations were determined with enzymatic methods (Bergmeyer, 1984) modified for a 96-well plate (Hammelman et al., 2003). All metabolites were analyzed at room temperature and measured spectrophotometrically at 340 nm. ATP, ADP, AMP, and IMP contents were quantified by high-performance liquid chromatography (HPLC). Breifly, samples were filtered (PTFE 0.22 μm pore size) then injected into an HP Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA). Separation was achieved using continuous gradient elution with two mobile phases using a C18 2.6 μm 50 × 4.6 mm column (Thermo Scientific, Pittsburgh, PA, USA). Flow rate was maintained at 1.25 ml/min. Detections were performed at room temperature at a wavelength of 260 nm (Bernocchi et al., 1994; Williams, Vidt, & Rinehart, 2008).

Statistical analysis

Metabolite and pH data collected at different time points were analyzed using the PROC MIXED procedure of SAS for repeated measures with *in vitro* model (tube) as the experimental unit. The statistical model for the first experiment included the main effects of mitochondria (0 or 0.5), inhibitor (with or without), time, and their interactions. In the second and third experiments, the model included the main effects of treatment, time, and their interaction. The 'repeated' statement was used for the repeated factor time. The slices statement was used to determine treatment effects at individual time points. Differences between mean values were tested using Bonferroni correction for multiple comparison. All data are presented as least-squares means ± SE.

Results and discussion

To evaluate the possible contribution of mitochondria in postmortem metabolism, we utilized a modified Scopes *in vitro* model that mimics muscle postmortem glycolysis (England et al., 2014; Scopes, 1973). The *in vitro* model consists of a buffer that contains all metabolites and cofactors required for glycolysis in addition to powdered pre-rigor muscle as the source of glycolytic enzymes. The rate and extent of glycolysis in the aforementioned model mimics closely that occurring *in vivo* (England, Matarneh, Scheffler, Wachet, & Gerrard, 2015; England et al., 2014). Using this approach, we eliminated sources of variation and confounding factors that often plague *in vivo* studies. We chose mitochondrial protein at a concentration of 0.5 mg/ml; a concentration comparable to that estimated for porcine *longissimus* muscle (Scheffler et al., 2015). The intactness of the isolated mitochondria used in the *in vitro* model was > 90%. A combination of inhibitors that block the activity of complexes I, IV, and V were used to evaluate the contribution of different mitochondrial compartments. For a better representation of postmortem metabolism, reaction vessels were sealed throughout the duration of the study to prevent the diffusion of air. Finally, pH, glycolytic metabolites, and adenine nucleotides were evaluated to explore the relationship between mitochondria and the flux through glycolysis.

During the conversion of muscle to meat, the accumulation of H^+ generated from the hydrolysis of ATP causes a drop in pH (Hamm, 1977). A significant three-way interaction was detected among mitochondria, inhibitor, and time for pH (P = 0.02, Fig. 3-1). Reaction vessels with mitochondria tended to have greater pH at 30 min (P = 0.09) in comparison to those without mitochondria or when mitochondria were combined with inhibitors. While there was no difference between treatments at 120 min (P = 0.3), the inclusion of mitochondria in the *in vitro* model lowered pH at 240 and 1440 min regardless of inhibitor ($P \leq 0.0003$).

Under postmortem anoxic conditions, pyruvate generated through glycolysis is reduced to lactate, which then accumulates in the muscle. Mitochondria influenced lactate over time (mitochondria × time, $P < 0.0001$, Fig. 3-2). In the absence of inhibitors, mitochondria significantly reduced lactate formation at 30 min ($P = 0.04$) compared to those without mitochondria or when mitochondria were combined with inhibitors. The difference in lactate between treatments was lost at 120 min ($P = 0.6$). However, greater lactate accumulation was observed at 240 and 1440 min ($P \leq 0.0003$) in treatments containing mitochondria (with or without inhibitors).

Stored muscle glycogen is degraded through glycogenolysis to yield G6P, which fuels the glycolytic pathway. No difference in glycogen degradation among treatments was detected at 30 min. However, when mitochondria were added to reactions, regardless of inhibitors, enhanced glycogen degradation occurred, as evidenced by lower glycogen from 120 to 1440 ($P \leq 0.05$; Fig. 3-3A) compared to samples without mitochondria. No difference in G6P concentration among treatments was observed at 30 min (Fig. 3-3B). At 120 and 240 min, samples with mitochondria (in presence or absence of inhibitors) had greater G6P ($P \leq 0.02$) compared to those without mitochondria. However, there was no significant difference in G6P between treatments at 1440 min. Results of the current study are similar to our previous study (Scheffler et al., 2015), where mitochondria promoted greater glycogen degradation and G6P accumulation *in vitro*.

In an attempt to maintain postmortem ATP levels, the enzyme adenylate kinase combines two molecules of ADP to from ATP and AMP. Subsequently, AMP is deaminated by AMP deaminase to IMP, which eventually leads to the depletion of the adenine nucleotides. Due to lack of significant differences in ADP content and the inability to detect AMP, ADP and AMP

abundances are not included herein. Mitochondria influenced ATP over time (mitochondria × time, $P = 0.0004$, Fig 3-4A). No difference in ATP was observed between treatments at 30 min. At 120 min, mitochondria regardless of inhibitor accelerated ATP loss ($P < 0.005$) compared to samples with inhibitor only, while controls were intermediate. Treatments containing mitochondria had lower ATP contents at 240 min ($P = 0.03$) compared to controls, while samples containing mitochondria in combination with inhibitors or with inhibitors alone were intermediate. ATP was below detection at 1440 min. Because IMP accumulation is dependent on ATP depletion, patterns of IMP accumulation were similar to ATP loss. IMP varied over time (mitochondria × time, $P = 0.004$, Fig. 3-4B). While there was no difference in IMP concentration between treatment groups at 30 min, reaction vessels with mitochondria (in presence or absence of inhibitors) accumulated more IMP at 120 min ($P = 0.03$) in comparison to those without mitochondria. No difference between treatments was detected at either 240 or 1440 min.

During early postmortem metabolism, skeletal muscle consumes oxygen stored in myoglobin for ATP production. The amount of postmortem ATP produced aerobically varies between 0.4 and 6.0 µmol/g, depending on the concentration of myoglobin in the muscle (Reviewed by Pösö & Puolanne, 2005). Once oxygen is depleted, glycogen is mobilized anaerobically to lactate and H^+, thereby causing a drop in pH. The trend toward greater pH coupled with lower lactate at 30 min (Fig. 3-1 and 3-2, respectively) in reaction vessels containing mitochondria and no inhibitor was likely due to aerobic metabolism. This is evidenced by lack of effect of mitochondria when combined with ETC inhibitors. Pyruvate oxidation by mitochondria reduces lactate formation by lowering the availability of pyruvate for lactate dehydrogenase (LDH); the enzyme responsible for converting pyruvate to lactate. This in

turn decreases the rate of pH decline due to the fact that aerobic metabolism is not associated with net H^+ production (Robergs, Ghiasvand, & Parker, 2004). While aerobic oxidation of glucose generates at least 10 times more ATP than anaerobic glycolysis, the lack of difference in ATP, IMP, glycogen, and G6P suggest that anaerobic glycolysis was the dominant pathway. Nonetheless, these data indicate that mitochondria can affect the rate of pH decline early postmortem by stabilizing ATP levels and reducing glycolytic flux. This may partly explain the lower incidence of pale, soft, and exudative conditions (PSE) in red oxidative muscle when compared to their white glycolytic counterparts.

The lack of difference between treatments in pH and lactate at 120 min indicates that mitochondrial respiration was either greatly diminished or terminated. This is probably due to the depletion of oxygen in the reaction vessels and/or to the loss of mitochondria functionality. Tang et al. (2005) reported that mitochondria integrity and functionality dramatically impaired as time progresses postmortem, mainly due to the drop in pH. Further, mitochondria can sequester large quantities of calcium, which can lead to the opening of mitochondrial permeability transition pore (mPTP) in the inner membrane. The formation of mPTP coupled with adenine nucleotide exhaustion destroys the proton motive force and results in mitochondrial dysfunction (reviewed by Halestrap & Pasdois, 2009). Although there was no difference in lactate at 120 min, it is important to note that samples containing mitochondria without inhibitor had greater net lactate production due to lower initial lactate concentration at 30 min. Similarly, at 240 and 1440 min mitochondria increased the flux through glycolysis, as evidenced by the significantly lower pH and greater lactate. Curiously, mitochondria produced the same effect in the presence of inhibitors, suggesting that the effect was through mechanisms independent of maintaining ATP levels. Flux through glycolysis is regulated by three rate-limiting enzymes: glycogen

phosphorylase, phosphofructokinase-1 (PFK-1), and pyruvate kinase. The activity of these enzymes dramatically increases in response to low energy charge (Greaser, 1986). In samples with mitochondria, ATP was lower at 120 and 240 min. Correspondingly, mitochondria enhanced glycogen degradation and lactate accumulation, indicating glycolysis was hastened to meet ATP demand. England et al., (2014) indicated that when glycogen is not a limiting factor, the extent of postmortem pH decline is mainly determined by the activity of PFK-1. The authors proposed that hastened glycolysis may increase the potential for extended glycolysis. Basically, rapid glycolysis allows for more substrate to pass PFK-1 before pH inactivation, thereby extending pH decline.

The average ultimate pH in reaction vessels containing mitochondria regardless of inhibitor was near 5.3 while in treatments without mitochondria was around 5.55. These data suggest that mitochondria added to an *in vitro* system may recapitulate the extended glycolysis that causes the acid meat condition known to occur in the AMPK$\gamma3^{R200Q}$ mutated pig. Pigs harboring this mutation possess greater mitochondrial content and oxidative capacity compared to non-mutant pigs (Scheffler et al., 2014). While AMPK$\gamma3^{R200Q}$ mutated pigs are widely recognized for greater glycogen content, we have recently shown that greater flux through glycolysis is the causative mechanism for extending glycolysis in the muscle of these pigs (Matarneh et al., 2015). Therefore, results from the present study suggest that mitochondria may contribute to the low ultimate pH observed in meat from these pigs. While the exact underlying mechanism is not clear, several possibilities exist for mitochondria to extend pH decline. First, mitochondria can accelerate glycolysis by increasing the rate of ATP hydrolysis. Hudson, (2012) proposed mitochondria can increase the rate of glycolysis as F_1F_0 ATP synthase can operate in the reverse direction and hydrolyze ATP. The author also suggests mitochondrial ATPase

postmortem activity may in part explain the faster glycolysis that sometimes occurs in red oxidative muscle. Second, mitochondria may shift the redox state in the cytosol toward greater NAD^+ / NADH ratio (Jong & Davis, 1983), and indirectly allow for greater flux through glycolysis. Third, mitochondria could provide glycolysis with metabolites and co-factors like calcium, inorganic phosphate, and adenine nucleotides, which in turn, could increase glycolytic flux.

To explore how mitochondria alter glycolytic flux, we tested whether mitochondria need to be functioning (intact). This was accomplished by mechanically disrupting mitochondria with a Polytron homogenizer. Mechanically disrupted mitochondria were centrifuged, and resulting supernatants were transferred to new tubes while pellets were re-suspended. Mitochondrial supernatants or pellets were then re-analyzed in the *in vitro* model to test which compartment was responsible for the effect. A significant treatment × time interaction was observed for pH ($P < 0.0001$, Fig. 3-5). While no difference in pH among treatments was observed at 30 min, samples containing mitochondrial supernatants had lower pH in comparison to those with mitochondrial pellets, while controls were intermediate. At 240 and 1440 min, reactions with mitochondrial supernatants reached a lower pH ($P \leq 0.0003$) compared to controls or those containing aliquots of mitochondrial pellets. Similarly, treatments influenced lactate over time (treatment × time, $P < 0.0001$, Fig. 3-6). Lactate level was not different for any treatment from 0 to 120 min. At 240 and 1440 min, reaction vessels with mitochondrial supernatants had augmented lactate levels compared to controls and those containing aliquots of re-suspended mitochondrial pellets ($P \leq 0.0004$). These data suggest that mitochondrial supernatants are responsible for producing the same glycolytic flux effect as preparations containing intact mitochondria at 240 and 1440 min. Intact mitochondria, however, are required for creating a

"shouldering effect" on flux through glycolysis that was observed at 30 min in the first experiment. Finally, our results suggest that the causative agent is water soluble, which can be a metabolite or a protein-based agent.

To further test the aforementioned and narrow the focus of our investigations, mitochondrial supernatants were deproteinized with PCA, neutralized, and then tested in the *in vitro* model. While proteins can be removed using this approach, metabolites are retained in the samples. Treatment differentially affected pH over time (treatment × time, $P < 0.0001$, Fig. 3-7). No difference between the three treatments was observed at 30 min. From 120 to 1440 min, samples containing mitochondrial supernatant had the lowest pH ($P \leq 0.0005$). The effect of mitochondrial supernatant was lost when samples were treated with PCA. Lactate was significantly affected by the interaction between treatment and time ($P < 0.0001$, Fig. 3-8). Although there was no difference between treatments at 30 min, samples with mitochondrial supernatants accumulated more lactate at 120 min ($P = 0.03$) compared to PCA treated samples, while controls were intermediate. At 240 and 1440, greater lactate content was detected when using samples containing mitochondrial supernatants ($P \leq 0.02$) compared to control or PCA-treated samples. These data show that the effect of mitochondrial supernatant was lost after PCA treatment and suggest a mitochondrial-based protein is capable of increasing glycolytic flux in postmortem muscle tissue. Conditions during postmortem metabolism are favorable for the formation of the mPTP. Indeed, the opening of mPTP leads to mitochondrial swelling and subsequently results in rupture of the mitochondrial membranes, thereby allowing the release of mitochondrial proteins that can affect glycolysis (reviewed by Halestrap & Pasdois, 2009).

Conclusions

Results presented herein indicate that the inclusion of mitochondria muscle to an *in vitro* model

system enhance glycolytic flux and pH decline and may be in part responsible for the acid meat development occurring in muscle from AMPK$\gamma 3^{R200Q}$ mutated pigs. Further, mitochondria accelerated ATP hydrolysis, glycogen degradation, and G6P accumulation. Mitochondrial intactness and functionality were not necessary for the induction of this effect. Additionally, our data revealed that a water soluble mitochondrial protein is the causative agent. Additional research is needed to identify this protein. Collectively, mitochondria can contribute to the determination of postmortem ultimate pH, contrary to popular dogma. To that end, we have updated our working model (England et al., 2014; England et al., 2015; Matarneh et al., 2015; England et al., 2016) of those factors controlling the rate and the extent of postmortem pH decline (Fig. 3-9).

Acknowledgments

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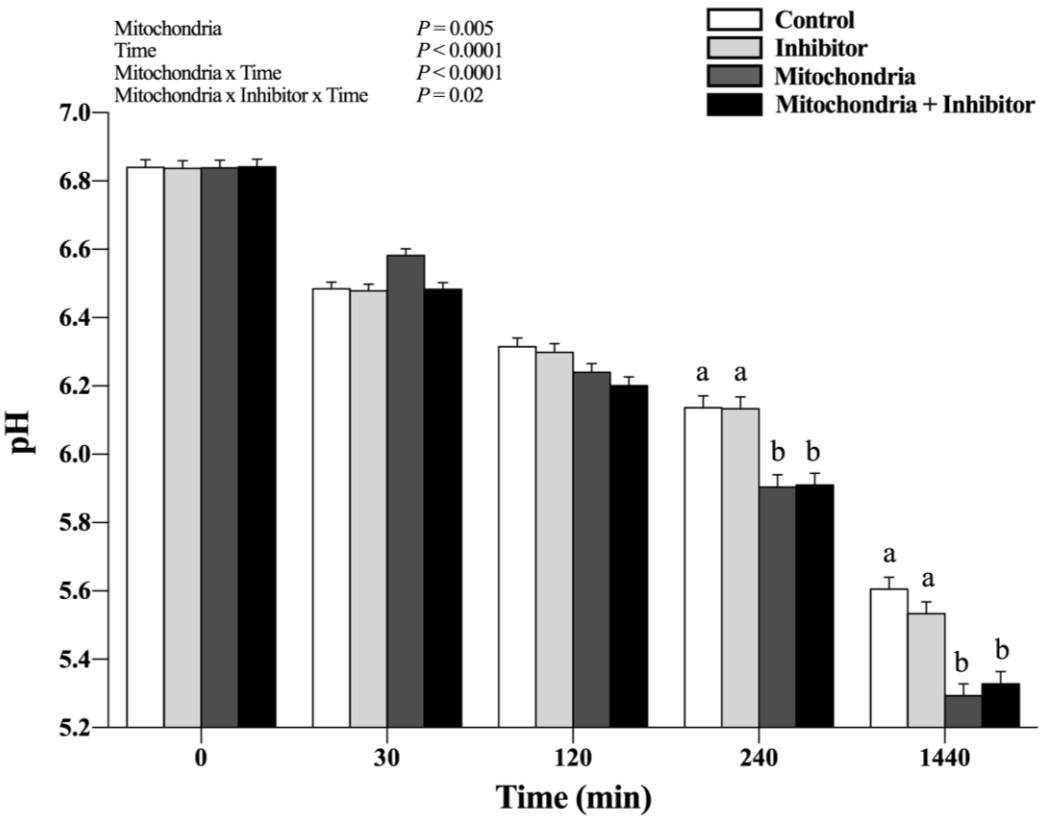

Fig. 3-1. Mean pH of the *in vitro* model. Data are LS means ± SE a,b means lacking a common letter differ within a time point ($P < 0.05$).

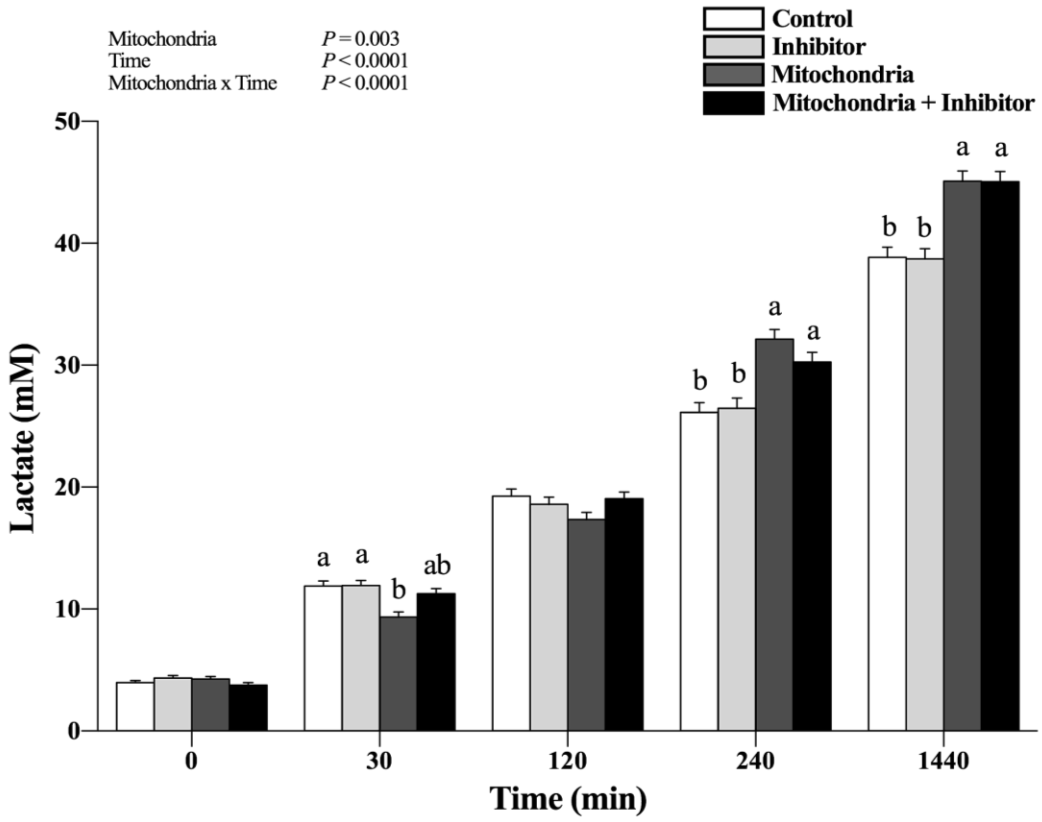

Fig. 3-2. Mean lactate (mM) of the *in vitro* model. Data are LS means ± SE. a,b means lacking a common letter differ within a time point ($P < 0.05$).

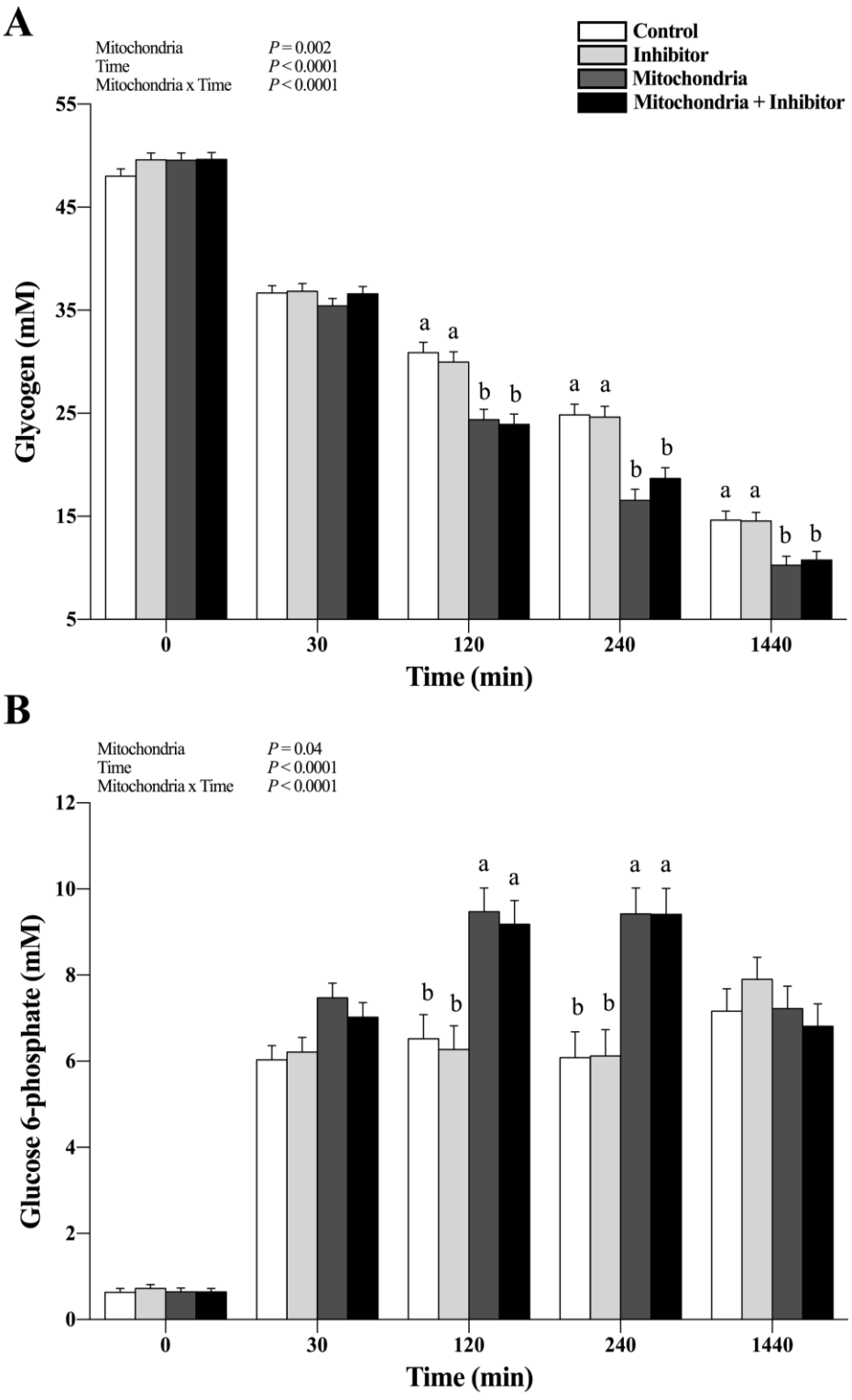

Fig. 3-3. Mean glycogen (mM; A), glucose 6-phosphate (mM; B) of the *in vitro* model. Data are LS means ± SE. a,b means lacking a common letter differ within a time point ($P < 0.05$).

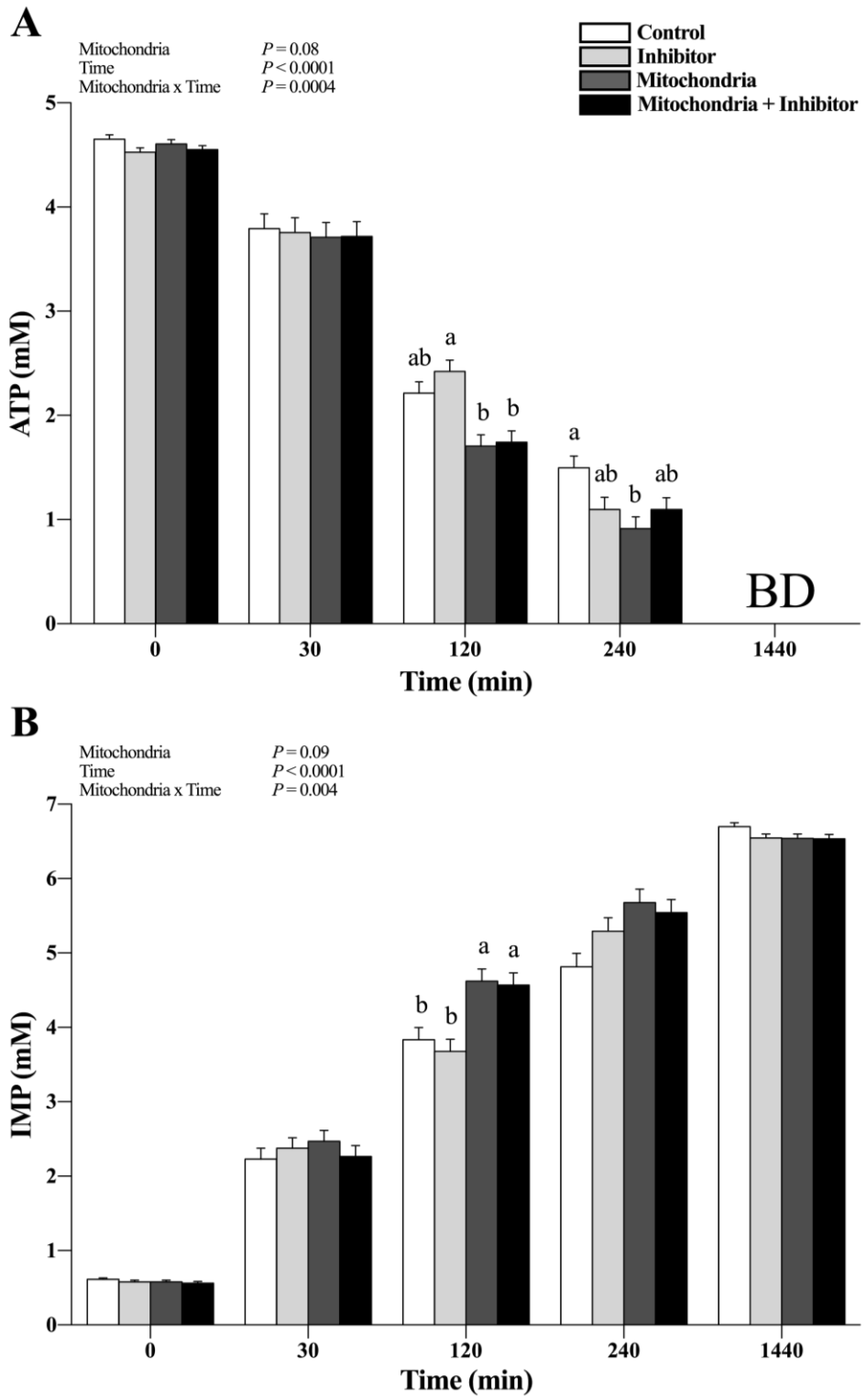

Fig. 3-4. Mean ATP (mM; A), IMP (mM; B) of the *in vitro* model. Data are LS means ± SE. a,b means lacking a common letter differ within a time point ($P < 0.05$). BD = below limit of detection.

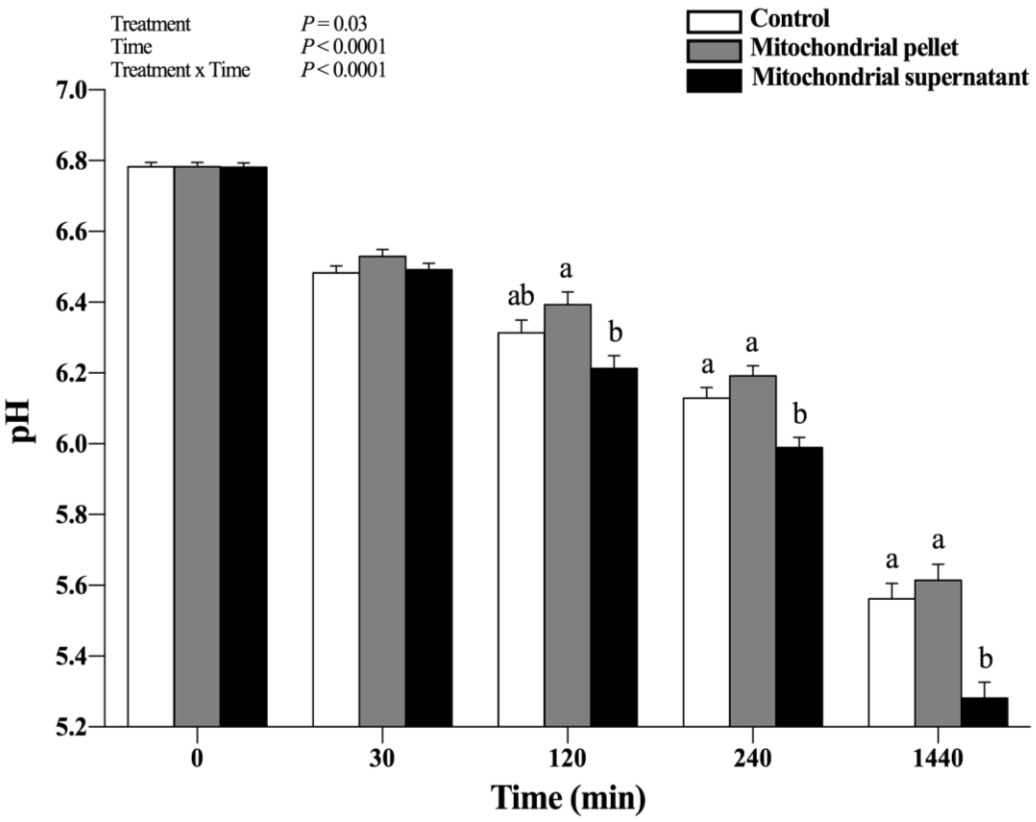

Fig. 3-5. Mean pH of the *in vitro* model. Data are LS means ± SE. a,b means lacking a common letter differ within a time point ($P < 0.05$).

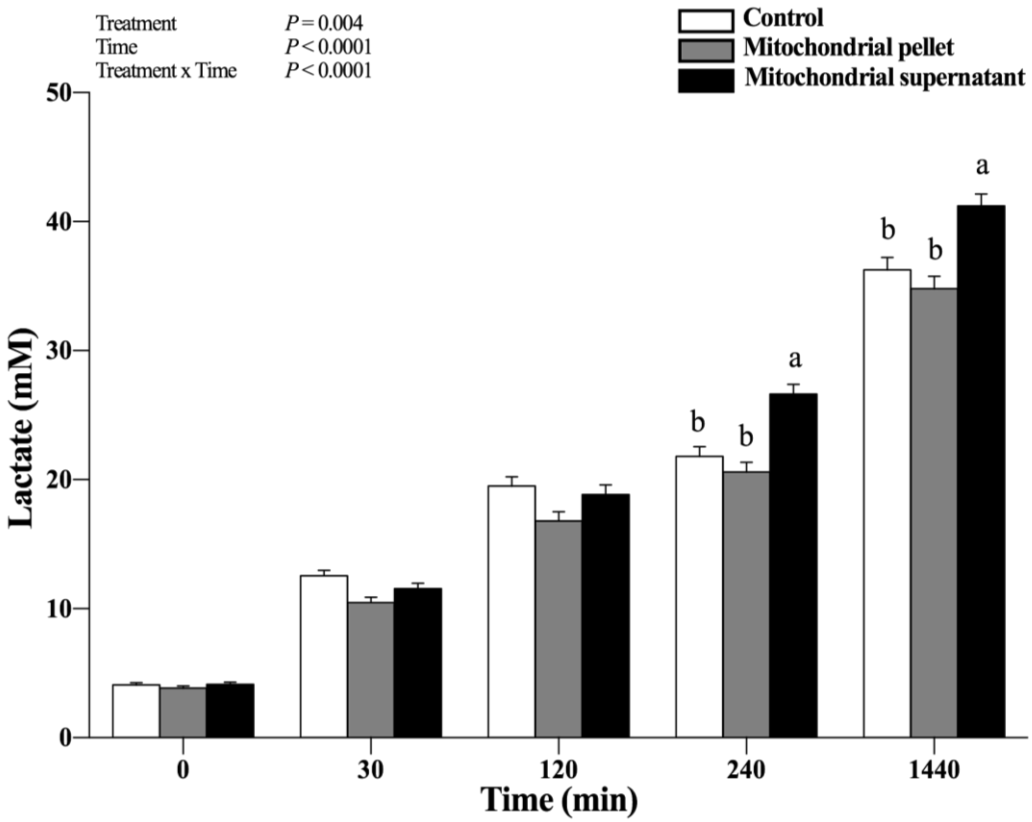

Fig. 3-6. Mean lactate (mM) of the *in vitro* model. Data are LS means ± SE. a,b means lacking a common letter differ within a time point ($P < 0.05$).

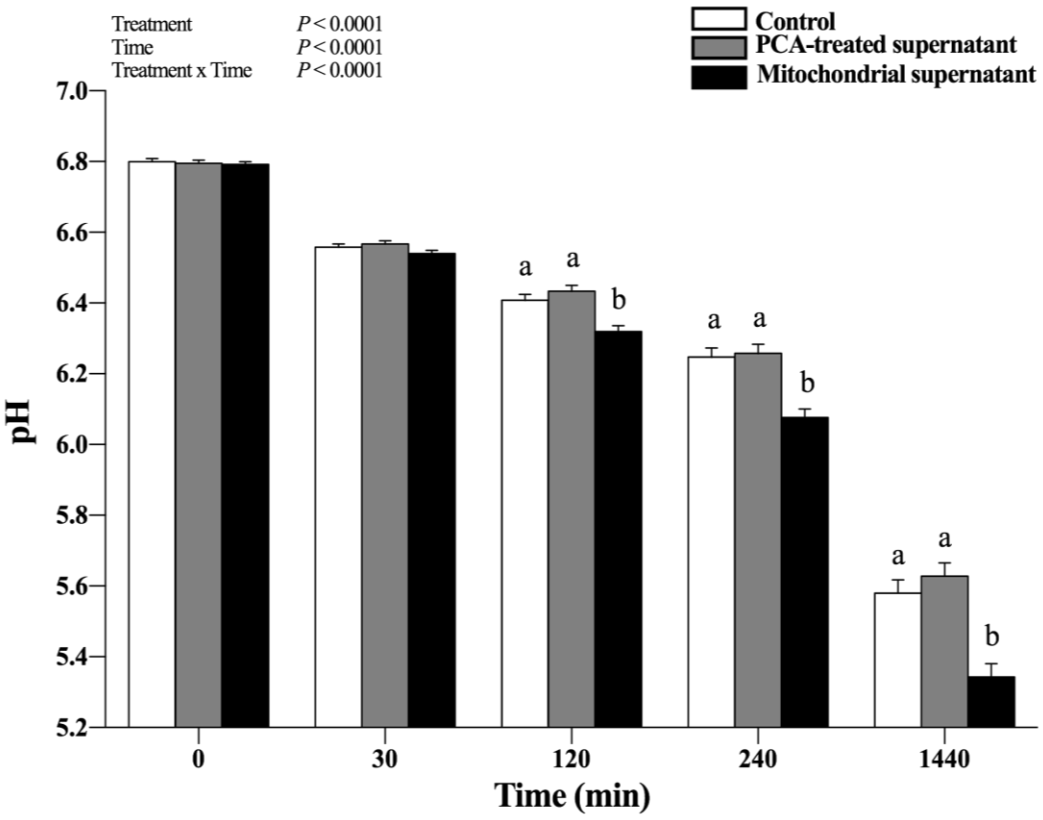

Fig. 3-7. Mean pH of the *in vitro* model. Data are LS means ± SE. a,b means lacking a common letter differ within a time point ($P < 0.05$).

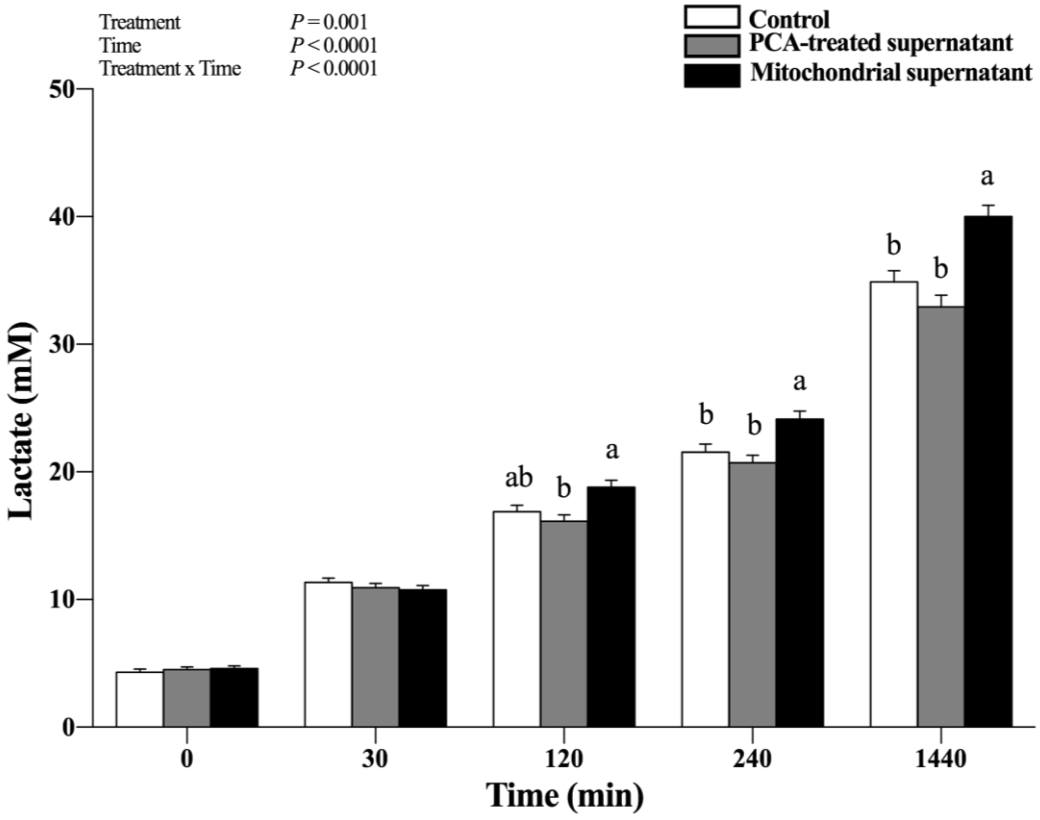

Fig. 3-8. Mean lactate (mM) of the *in vitro* model. Data are LS means ± SE. a,b means lacking a common letter differ within a time point ($P < 0.05$).

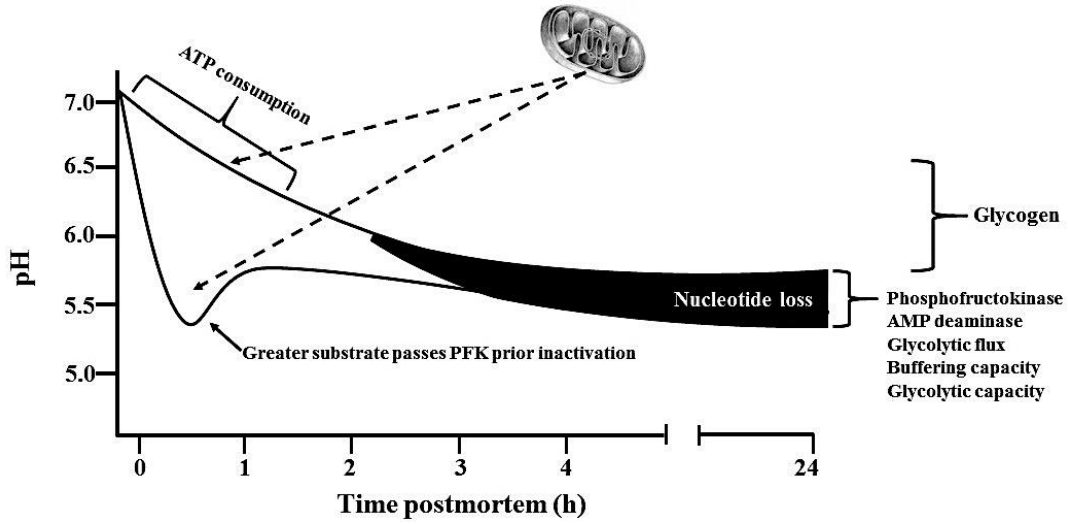

Fig. 3-9. Working model of the factor controlling the rate and extent of postmortem metabolism.

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Chapter 4 – Mitochondrial F_1-ATPase is responsible for increased glycolytic flux

Abstract

The experiment was conducted in an attempt to identify the mitochondrial protein responsible for enhancing glycolytic flux that we previously reported. We hypothesized that the F_1 domain of mitochondrial F_1F_0 ATP synthase promotes ATP hydrolysis and thereby the flux through glycolysis. Mitochondria were incorporated into an *in vitro* system designed to recapitulate postmortem glycolysis with or without Na-azide to specifically inhibit the β-subunit of mitochondrial F_1 domain that catalyzes ATP hydrolysis. Addition of mitochondria enhanced ATP hydrolysis, glycogen degradation, lactate accumulation, and pH decline in the *in vitro* system. However, the majority of mitochondria-mediated enhancement in glycolytic flux was abolished in the presence of Na-azide. To investigate further, myofibrillar and mitochondrial proteins were added to the *in vitro* system after 240 min from the initiation of the reaction. Greater pH decline and lactate accumulation were observed in system containing mitochondrial protein compared to their myofibrillar counterpart. In conclusion, mitochondrial F_1 domain is capable of increasing glycolytic flux through promoting greater ATP hydrolysis at lower pH.

Key words: Mitochondrial F_1-ATPase; Glycolytic flux; Ultimate pH; Acid meat.

Introduction

During the process of converting muscle to meat, pH declines due to an accumulation of hydrogen ions (H^+) in the muscle. This process is, in fact, necessary to impart a unique set of characteristics on fresh meat, namely its ability to reflect light (color) and retain moisture (water holding capacity) (Scheffler & Gerrard, 2007). While acidification of muscle via anaerobic

glycolysis postmortem is indisputable, the biochemical mechanisms responsible for pacing this proton-mediated decline or arresting the process in such a metabolically complex tissue remain rather obscure. This lack of clarity has literally stalled, or rendered futile, attempts to develop technologies to predict meat quality early in the harvesting process so that greater product consistency can be managed through sorting operations (Henckel, Karlsson, Jensen, Oksbjerg, & Petersen, 2002; Monin & Sellier, 1985; Scheffler, Scheffler, Kasten, Sosnicki, & Gerrard, 2013). Though difficult to fathom, this lack of progress is likely due, in part, the oversimplification of postmortem metabolism and how it is controlled.

Traditionally, postmortem muscle acidification has been attributed to the mobilization of stored muscle glycogen to lactate and H^+ by anaerobic glycolysis. While muscle tissue undeniably experiences a loss of oxygen during the postmortem period, we have shown in multiple studies that mitochondria can significantly contribute to the rate and extent of postmortem metabolism (England et al., 2016; Matarneh, England, Scheffler, Yen, & Gerrard, 2017; Scheffler, Matarneh, England, & Gerrard, 2015). In our most recent study (Matarneh et al., 2017), we used an *in vitro* system to evaluate the contribution of mitochondria to postmortem metabolism. Our data revealed that intact mitochondria initially slowed (shouldered) the rate of pH decline by stabilizing ATP levels and reducing glycolytic flux. Afterward, however, mitochondria can also enhance glycogen degradation, ATP hydrolysis, lactate accumulation, and pH decline. After a series of experiments, we found that the causative agent for this enhanced glycolytic flux is related to or closely associated with mitochondria. Therefore, the present study attempted to identify this causative protein.

Mitochondrial ATP synthase is composed of two functional domains: the membrane-embedded domain, F_0, which is responsible for proton translocation across mitochondrial inner

membrane, and the soluble catalytic domain, F_1, that extends into the matrix. During cellular respiration, protons are pumped from the matrix to the intermembrane space to generate a proton gradient across the inner membrane (proton motive force). The flow of protons back into the matrix through the F_1F_0 ATP synthase drives the synthesis of ATP. However, the increase in mitochondrial Ca^{2+} and reactive oxygen species (ROS) levels, similar to that occurring in postmortem muscle, promote the formation of a mitochondrial permeability transition pore (mPTP) (Halestrap & Pasdois, 2009; Lomiwes, Farouk, Wiklund, & Young, 2014). This event essentially destroys the proton motive force across the inner membrane, thereby forcing the ATP synthase to function in reverse, consuming ATP in an attempt to conserve the proton motive force (St-Pierre, Brand, & Boutilier, 2000). Additional consumption of ATP by mitochondria has been proposed to promote postmortem metabolism (Hudson, 2012). Yet, our previous study (Matarneh et al., 2017) revealed that enhanced glycolytic flux was maintained in the presence of oligomycin, an inhibitor of ATP synthase that inhibits both ATP synthesis and hydrolysis. Even so, however, this does not rule out ATP synthase from being the causative agent, as the F_1 domain can dissociate and become oligomycin insensitive and act only as ATPase (F_1-ATPase) (Dubinsky, 1987; Foster & Fillingame, 1979; Gibson, 1983). Therefore, we hypothesized that mitochondrial F_1-ATPase, more specifically its β-subunit, increases ATP hydrolysis and subsequently the flux through glycolysis. To test our hypothesis, we utilized Na-azide to inhibit the ATPase activity of the F_1 domain (Ishii, Shirai, Makino, & Nishikata, 2014). We expected that if F_1-ATPase is the causative protein, then including Na-azide would reverse the observed effects.

Materials and Methods

Muscle sampling

A total of 10 market-weight crossbred pigs (100-125 kg, Yorkshire × Duroc, mixed sex) were slaughtered in Virginia Tech Meat Science Center using standard commercial procedures. Muscle samples were collected from the *longissimus lumborum* muscle within 5 min of exsanguination. Samples were used for mitochondria and myofibril isolation or immediately snap frozen in liquid nitrogen, and stored at − 80 °C to be used for the *in vitro* assay.

Mitochondria isolation

Mitochondria isolation was performed using the differential centrifugation method as outlined by Scheffler et al. (2014) with minor modification. All steps of the mitochondria isolation procedure were performed at 4 °C. Briefly, muscle samples were finely minced with scissors in ice-cold isolation buffer (5 ml/g of muscle; 100 mM sucrose, 180 mM KCl, 50 mM Tris, 5 mM $MgCl_2$, 10 mM EDTA, 1 mM K-ATP, pH 7.4). Tissue suspensions were treated with a protease (subtilisin A, 0.4 mg/ml) before homogenization with a Potter-Elvehjem type homogenizer system (Glas-Col, Terre Haute, IN. USA). Homogenates were diluted with isolation buffer to achieve ~ 20 ml/g of tissue followed by filtering through cheesecloth. Filtered homogenates were centrifuged at 1,000 × g for 10 min, and resulting supernatants were filtered again before a second centrifugation at 8,000 × g for 10 min. Mitochondrial pellets were re-suspended by repeated pipetting in mitochondrial suspension buffer (220 mM mannitol, 70 mM sucrose, 10 mM Tris–HCl, 1 mM EGTA, pH 7.4). Mitochondrial protein concentration was determined with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL. USA).

Myofibril isolation

Myofibril isolation was performed according to the procedures of Swartz, Greaser, & Marsh (1993) with slight modifications. All steps were performed at 4 °C. Muscle bundles, ~ 6 cm in length, were dissected, stretched, attached to applicator sticks, and bathed for 24 h with stirring

in ice-cold rigor buffer (75 mM KCl, 10 mM imidazole (pH 7.2), 2 mM $MgCl_2$, 2 mM EGTA, 1 mM NaN_3) in addition to 0.5% Triton X-100 and 0.1 mM PMSF. Following, muscle bundles were minced with a scalpel and homogenized in 15 volumes of rigor buffer for two 15-s bursts using (Polytron PT-MR 2100, Kinematica AG, Switzerland). Tissue homogenates were diluted with additional 5 volumes of rigor buffer before further homogenization with a Potter-Elvehjem type homogenizer system (Glas-Col, Terre Haute, IN. USA). Suspensions were then centrifuged at 1,000 × g for 10 min, and resulting pellet were suspended in 20 volumes of rigor buffer before homogenized again for three 5-s bursts. Suspensions were recentrifuged, pellets were resuspended in rigor buffer, and rehomogenized 2 more times. To the suspension, 10 more volumes of rigor buffer containing 0.5% Triton X-100 were added before filtering through cheesecloth. Myofibrils were collected by centrifugation of the suspension at 1,000 × g for 10 min. Myofibril pellets were then washed three more times with 20 volumes of rigor buffer and centrifuged at 1,000 × g for 10 min. After the final washing, myofibril pellets were thoroughly resuspended to approximately 5 mg/ml in rigor buffer. For myofibrillar protein determination, myofibrillar samples were mixed with an equal volume of solubilization buffer (8 M urea, 2 M thiourea, 3% SDS (wt/vol), 75 mM dithiothreitol, and 50 mM Tris-HCl, pH 6.8) (Warren, Krzesinski, & Greaser, 2003) and heated at 90 °C for 10 min. Protein concentration was determined using the RC DC protein assay kit (BioRad Laboratories, Hercules, CA, USA).

SDS-PAGE and immunoblotting

Mitochondrial samples were homogenized with a Polytron homogenizer then centrifuged at 13,000 rpm for 5 min at 4 °C. Supernatants were transferred to new tubes while pellets were re-suspended again in the mitochondrial suspension buffer. Total mitochondrial preparations, and mitochondrial supernatants and pellets were solubilized with a buffer containing 8 M urea, 2 M

thiourea, 3% SDS (wt/vol), 75 mM dithiothreitol, and 50 mM Tris-HCl, pH (6.8), and 0.03% bromophenol blue (wt/vol) (Warren et al., 2003). Equal amounts of protein were loaded and separated by SDS-PAGE (10% acrylamide), transferred to nitrocellulose membrane, blocked for an hour at room temperature, and immunoblotted overnight at 4 °C with primary antibody specific for β-subunit of mitochondrial ATP synthase (Abcam, Cambridge, MA, USA). Bands were visualized with IRDye fluorescent secondary antibody (LI-COR Biosciences, Lincoln, NE) and intensities were quantified using LI-COR Biosciences Odyssey imaging system and software (Li-Cor, Inc., Lincoln, NE, USA).

In vitro glycolysis model

Frozen *longissimus lumborum* muscle samples were powdered under liquid nitrogen and homogenized at a 100 mg/ml into a reaction buffer containing 40 mM glycogen, 60 mM KCl, 5 mM $MgCl_2$, 10 mM Na_2HPO_4, 30 mM creatine, 25 mM carnosine, 10 mM sodium acetate, 5 mM ATP, 0.5 mM ADP, and 0.5 mM NAD^+ (pH 7.4) (England, Matarneh, Scheffler, Wachet, & Gerrard, 2014; Scopes, 1973). Treatments were: control (only the *in vitro* system), mitochondria (0.5 mg/ml mitochondria were incorporated into the *in vitro* system), Na-azide (500 µM Na-azide), and mitochondria + Na-azide (0.5 mg/ml mitochondria + 500 µM Na-azide). Aliquots were removed at 0, 120, and 1440 min for pH and metabolite analysis. Reaction vessels were maintained at 25 °C for the duration of the trial. In this study, six separate *longissimus lumborum* muscles were used but powdered muscle from each *longissimus lumborum* muscle was split between treatments.

In another experiment, myofibrillar and mitochondria proteins were added to the *in vitro* system at 0.5 mg/ml after 240 min from the start of the experiment. Samples were removed at 0, 240, and 1440 min for pH and lactate measurements. In this study, four separate *longissimus*

lumborum muscles from four different pigs were used but powdered muscle from each *longissimus lumborum* was split between treatments.

pH determination

Aliquots for pH analysis were mixed with buffer containing 25 mM Na-iodoacetate and 750 mM KCl (pH 7.0) at 4:1 (vol/vol) (Bendall, 1973). Samples were then centrifuged at 13,000 rpm for 5 min at room temperature, equilibrated to 25 °C, and measured directly using an Orion Ross Ultra pH glass electrode (Thermo Scientific, Pittsburgh, PA, USA).

Metabolite analysis

Aliquots for glycogen analysis were mixed with 2.5 M HCl at 1:1 ration, heated at 90 °C for 2 h, centrifuged at 13,000 rpm for 5 min, and resulting supernatants were neutralized with 1.25 M KOH (Bergmeyer, 1984). Aliquots for glucose 6-phosphate, glucose, lactate, ATP, and inosine monophosphate (IMP) were mixed with an equal volume of 1 M perchloric acid and incubated on ice for 20 min. Following centrifugation at 13,000 rpm for 5 min, supernatants were transferred to new tubes and neutralized with 2 M KOH (Bergmeyer, 1984). Glycogen, glucose, glucose 6-phosphate, and lactate concentrations were determined with enzymatic methods modified for a 96-well plate (Hammelman et al., 2003). All metabolites were analyzed at room temperature and measured spectrophotometrically at 340 nm. ATP and IMP contents were quantified using HP Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) according to Bernocchi et al. (1994) and Williams, Vidt, & Rinehart (2008). Briefly, samples were filtered (PTFE 0.22 μm pore size) then injected into a C18 2.6 μm 50 × 4.6 mm column (Thermo Scientific, Pittsburgh, PA, USA). Flow rate was maintained at 1.25 ml/min. Detections were performed at room temperature at a wavelength of 260 nm.

Statistical analysis

Data were analyzed as a split plot design using the mixed model of JMP (SAS Institute Inc., Cary, NC). The statistical model included the main effect of treatment and time and their interaction, with *in vitro* models (tubes) considered as the main plots and times as subplots. The slice function was used to determine treatment effects at each time points. Differences between means were evaluated using a Student's t-test, with $P \leq 0.05$ considered statistically significant. All data are expressed as least-squares means ± SE.

Results and discussion

In our previous studies (Matarneh et al., 2017), mitochondria were mechanically disrupted and separated into supernatant and pellet fractions by centrifugation. The causative agent was shown to be a protein that resides in the supernatant (soluble). In the present study, the β-subunit of mitochondrial F_1-ATPase was chosen as a candidate, primarily because it is a water-soluble protein that has the potential to affect glycolysis through increasing the rate of ATP hydrolysis (Hudson, 2012). In order for our hypothesis to be correct, the β-subunit must reside in the supernatant fraction. Therefore, western blot analysis was used to confirm the presence of the β-subunit in the supernatants. The difference in β-subunit abundance between total mitochondrial preparation, supernatants, and pellets is readily apparent in Fig. 4-1. The supernatant fraction contained more than 8 times greater ($P < 0.0001$) β-subunit content compared to that of pellet fraction. These data affirm that the F_1-ATPase was dissociated from the mitochondrial inner membrane and re-localized in the soluble fraction.

To test the role of mitochondrial F_1-ATPase in postmortem metabolism, we utilized our previously described *in vitro* system that mimics postmortem glycolysis (England et al., 2014; Matarneh et al., 2017). Either 0 or 0.5 mg/ml isolated mitochondria were incorporated into the *in vitro* system in the presence or absence of Na-azide. Na-azide is a potent noncompetitive

inhibitor that tightly traps ADP at the catalytic site in the β-subunit of F_1-ATPase. This trapping stabilizes the ADP-inhibited state of the enzyme, thereby preventing its ATPase activity (Bowler, Montgomery, Leslie, & Walker, 2006; Ishii et al., 2014; Vasilyeva, Minkov, Fitin, & Vinogradov, 1982). The pH of the *in vitro* system was significantly affected by the interaction between treatment and time ($P < 0.0001$; Fig. 4-2). No difference in pH between treatments was detected through 120 min. Yet, at 1440 min, samples containing only mitochondria had the lowest pH value ($P < 0.0001$), while control and Na-azide samples were the highest. When mitochondria were combined with Na-azide, however, pH decline was attenuated, as evidenced by the elevated pH value at 1440 min in comparison to that of mitochondria only treatment. These results are in agreement with our previously published findings where the inclusion of mitochondria to an *in vitro* model extended pH decline (Matarneh et al., 2017). Na-azide was responsible for about 65% inhibition of mitochondria-induced pH decline. At first glance, these results may seem to contradict our previously published findings (Matarneh et al., 2017), where mitochondria-induced glycolytic flux was maintained in the presence of oligomycin. Oligomycin is an inhibitor that blocks the passage of H^+ through the F_0 domain, and therefore preventing both the forward and reverse functions of F_1F_0 ATP synthase. However, the F_1 domain can dissociate and becomes oligomycin insensitive and exhibits a high level of ATPase activity (Dubinsky, 1987; Feinstein & Moudrianakis, 1984). It has been shown that mitochondria exhibit severe fragmentation (Khacho et al., 2014), and ATP synthase dissociation under low pH conditions was similar to that occuring postmortem.

To continue defining the relationship between mitochondrial F_1-ATPase and the flux through glycolysis, lactate, glycolytic metabolites, ATP, and IMP were measured in the *in vitro* system. During postmortem metabolism, pyruvate generated through glycolysis is reduced to

lactate by lactate dehydrogenase. This reaction is coupled with the oxidation of NADH to NAD^+ that is required for the GAPDH reaction, thereby allowing glycolysis to continue under anaerobic conditions. Treatment differentially affected lactate concentration of the *in vitro* system over time (treatment × time, $P = 0.0005$, Fig. 4-3). While no difference in lactate was detected at 120 min, samples containing mitochondria without Na-azide had the highest lactate level at 1440 min ($P = 0.002$) in comparison to the rest of the treatments.

In a futile attempt to maintain ATP levels, postmortem muscle mobilizes its glycogen reserves to yield glucose 6-phsphate that proceeds through glycolysis. Glycogen degradation was affected by the interaction between treatment and time ($P < 0.0001$; Fig. 4-4A). Significantly lower glycogen ($P = 0.001$) was observed in samples containing mitochondria without Na-azide at 120 min compared to the rest of the treatments. At 1440 min, samples containing only mitochondria had lower glycogen ($P = 0.05$) than that of control, while samples containing Na-azide and mitochondria + Na-azide were intermediate. There was also a significant interaction of treatment × time for mean glucose 6-phosphate concentration of the *in vitro* system ($P < 0.0001$; Fig. 4-4B). At 120 min, glucose 6-phosphate was greater in samples containing mitochondria regardless of Na-azide ($P < 0.0001$) compared to those without mitochondria. However, at 1440 min the opposite was true, where samples containing mitochondria possessed lower glucose 6-phosphate levels ($P < 0.0001$).

During the transformation of muscle to meat, ATP is hydrolyzed by muscle ATPases to ADP and eventually to IMP by adenylate kinase and AMP deaminase-catalyzed reactions. Once formed, IMP accumulates in the muscle and no longer contributes to ATP synthesis. At 120 min Samples containing mitochondria without Na-azide had the lowest ATP concentration ($P = 0.03$; Fig. 4-5A) compared to the rest of the treatments, while 1440 min ATP was below detection.

Samples containing only mitochondria accumulated more IMP at 120 min (P = 0.02; Fig. 4-5B) compared to the other treatments. Combined, lower ATP and greater IMP in system containing mitochondria indicate that ATPase activity was enhanced. However, when combined with Na-azide, mitochondria-induced ATP hydrolysis was diminished, which shows the efficacy of Na-azide treatment in our system. While Na-azide can inhibit other ATPases (Bowler et al., 2006; Vasilyeva & Forgac, 1998), myofibrillar ATPase (myosin ATPase) does not appear to be impacted, as evidenced by the lack of difference in pH between control and Na-azide treatments. Scopes (1974) showed that the rate of postmortem metabolism is dictated by the rate of ATP hydrolysis. The author also indicated that mechanisms controlling the flux through glycolysis must be determined by the rate of ATP hydrolysis. On the other hand, our lab has recently shown that postmortem PFK-1 activity rapidly decreases as muscle pH declines until complete inactivation is reached at pH 5.5 (England et al., 2014). Hastened glycolysis appears to increase the potential for extended pH decline, as it allows for more substrate to pass PFK-1 before it becomes inactivated. This notion is supported by our findings where enhanced ATP hydrolysis was coupled with greater glycogen degradation, lactate accumulation, and pH decline, suggesting that glycolysis was hastened to meet ATP demand. It is important to note that while mitochondria-enhanced ATP hydrolysis was completely abolished by Na-azide (Fig. 4-5A), the inhibitor did not completely reverse the effect of mitochondria on pH decline (Fig. 4-2). This suggests that additional mitochondrial protein(s) may account for part of the observed effect. Jong & Davis (1983) reported that mitochondria can increase the NAD^+/NADH ratio in the cytosol. Indeed, cytochrome c-mediated reduction of metmyoglobin coupled with oxidation of NADH to NAD^+ has been previously suggested (Tang, Faustman, Mancini, Seyfert, & Hunt, 2005). The increase in NAD^+ concentration may provide more substrate for GAPDH, allowing

for greater glycolytic flux. Regardless, these results confirm that mitochondria F_1-ATPase can significantly contribute to postmortem glycolysis.

Consistent with our previous findings (Matarneh et al., 2017), the effect of mitochondria on glycolytic flux required 120 min to be observed, suggesting that the effect may be cumulative over time. Another alternative explanation is that ATPase activity in the *in vitro* system was not limiting during the first 120 min, but as the pH declines it may become limiting, then additional ATPase activity by mitochondrial F_1-ATPase would enhance ATP hydrolysis and flux trough glycolysis. Because myosin is the most abundant protein in skeletal muscle, myosin ATPase is responsible for the majority of postmortem ATP hydrolysis (Hamm, Dalrymple, & Honikel, 1973). Similar to PFK-1, however, the activity of myosin ATPase is pH dependent (Bowker, Grant, Swartz, & Gerrard, 2004), where the activity substantially drops as pH declines from 6.5 to 5.5, at which complete inactivation is achieved. On the other hand, mitochondrial F_1- ATPase retains about 50% relative activity between pH 6 and 5 (Feinstein & Moudrianakis, 1984). To address this issue, myofibrillar and mitochondrial proteins were added to the *in vitro* system after 240 min from the initiation of the experiment. Myofibrillar protein was used to verify whether mitochondria-mediated ATP hydrolysis is a function of increased ATPase abundance or through maintaining a greater activity at a lower pH. Samples containing mitochondria had lower pH at 1440 ($P < 0.0001$; Fig. 4-6) than those from control and myofibril samples, while no difference between control and myofibrillar treatment was observed. Lactate followed the same trend as pH, where samples with mitochondria had greater lactate concentration ($P < 0.0001$; Fig. 4-7) than those containing myofibrillar protein. The lack of difference between control and myofibril treatments argues that ATPase activity rather than abundance was limiting at lower pH. Further, the addition of mitochondria after 240 min produced a similar effect to those added at 0 min,

suggesting that mitochondrial ATPase activity plays an important role at lower pH. Based on these results, we suggest that mitochondria can extend postmortem metabolism through maintaining greater ATPase activity at lower pH which, in turn, enhances flux through glycolysis.

These results must also be addressed in light of porcine AMPKγ_3^{R200Q} (RN^-) mutation. Pigs harboring this mutation produce meat with an abnormally low ultimate pH (pH < 5.4), usually referred to as acid meat (Copenhafer, Richert, Schinckel, Grant, & Gerrard, 2006; Lundström, Enfält, Tornberg, & Agerhem, 1998; Milan et al., 2000). This extremely low pH is very close to the isoelectric point of major muscle proteins, thereby causing greater exudate and a lighter colored product, quite unsavory to most consumers (Copenhafer et al., 2006; Scheffler, Park, & Gerrard, 2011). We have extensively used AMPKγ_3^{R200Q} pigs as a model for exaggerated pH decline because we believe that understanding how ultimate pH is breached in their muscle may help us prevent it from occurring in normal muscle. The low ultimate pH has been attributed to the high muscle glycogen of these pigs (Monin & Sellier, 1985). However, we and others have shown that additional substrate above a critical threshold contributes little to additional glycolytic flux (England et al., 2016; Henckel et al., 2002; Lundström et al., 1998; Scheffler et al., 2013). Instead, we have recently shown that low ultimate pH of muscle from AMPKγ_3^{R200Q} pigs is mainly due to increase in glycolytic flux, and to a lesser extent to low buffering capacity (Matarneh, England, Scheffler, Oliver, & Gerrard, 2015). Therefore, the underlying mechanism for this condition must work through increasing glycolytic flux. England, Matarneh, Scheffler, Wachet, & Gerrard (2015) showed that muscle from these mutant pigs had greater activity and abundance of the enzyme AMPD, which results in greater AMP levels postmortem. AMP is a potent activator for rate limiting enzymes in the glycolytic pathway (Greaser, 1986), which in

turn allows for extended postmortem glycolysis. Additionally, muscle from AMPKγ_3^{R200Q} pigs exhibit greater mitochondrial content and enhanced oxidative capacity compared to wild-type pigs (Estrade, Ayoub, Talmant, & Monin, 1994; Scheffler et al., 2014). Therefore, results of the current study strongly arguing that mitochondria can play a significant role in determining ultimate pH of the AMPKγ_3^{R200Q} pigs. Curiously, however, red muscle (high mitochondria) does not experience this problem but lacks elevated glycolytic enzymes (England et al., 2016). Collectively, lower ultimate pH of muscle from the AMPKγ_3^{R200Q} pigs is likely a function of lower AMP deaminase activity, lower buffering capacity, and greater mitochondrial content.

Implications

The results of these studies demonstrate that mitochondria can significantly contribute to postmortem metabolism. Mitochondria enhanced glycogen degradation, lactate accumulation, and pH decline. Our data also indicated that the majority of mitochondrial effect was through increasing the rate of ATP hydrolysis. Further, mitochondria maintained their effect when added after 240 min, while myosin ATPase failed to produce similar effect. These data expand our knowledge of how ultimate pH is determined and may change the way postmortem metabolism classically viewed. Additionally, the accuracy of modelling to predict postmortem pH decline might be improved by incorporation of the abundance of mitochondria in the model. Finally, AMPKγ_3^{R200Q} pig *longissimus* muscle contains greater mitochondria abundance than wild-type pigs, which may contribute to the lower ultimate pH muscle from these animals. To that end, we have updated our working model (England et al., 2015; England et al., 2016, 2014; Matarneh et al., 2017; Matarneh et al., 2015) of those factors controlling the rate and the extent of postmortem pH decline (Fig. 4-8).

Acknowledgments

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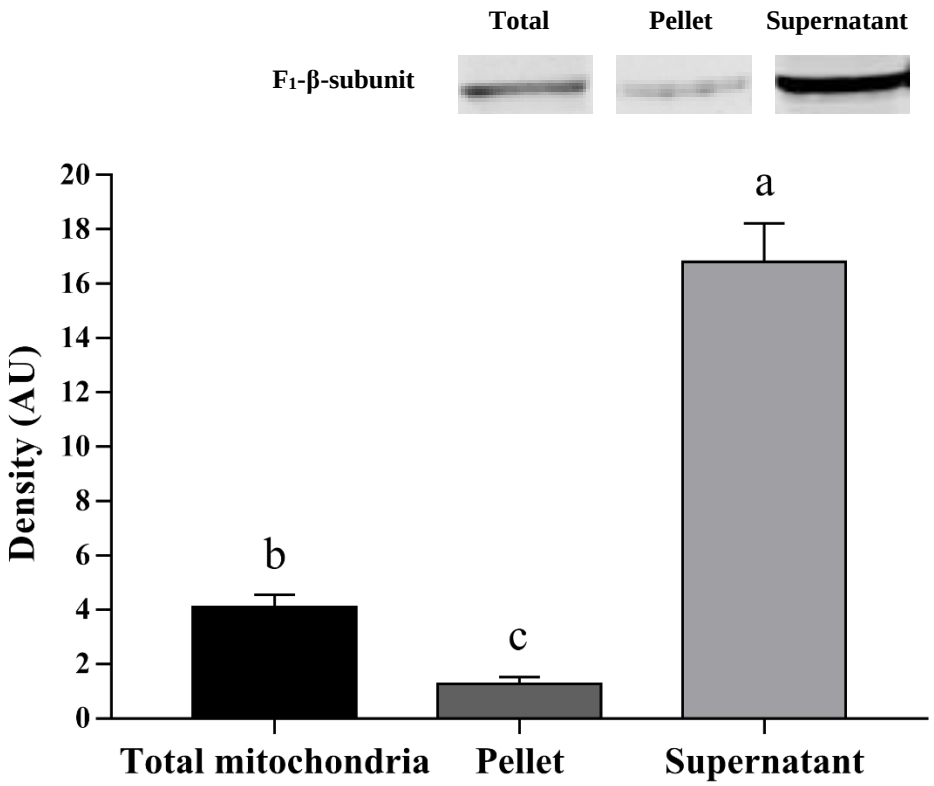

Fig. 4-1. Representative western blot of mitochondria F_1-ATPase β-subunit from total mitochondria, mitochondrial pellet, and mitochondrial supernatant (upper). Western blot analysis of F_1-ATPase β-subunit (bottom). Data are LS means ± SE. a,b,c means lacking a common letter differ ($P < 0.05$).

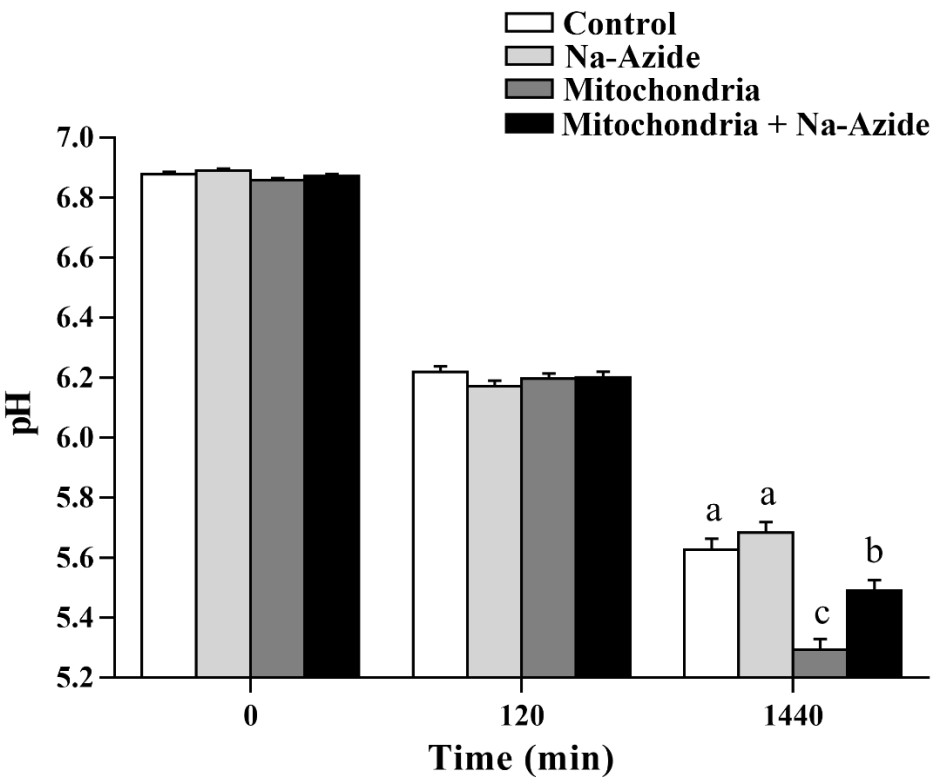

Fig. 4-2. Mean pH of the *in vitro* model. Data are LS means ± SE. a,b,c means lacking a common letter differ within a time point ($P < 0.05$).

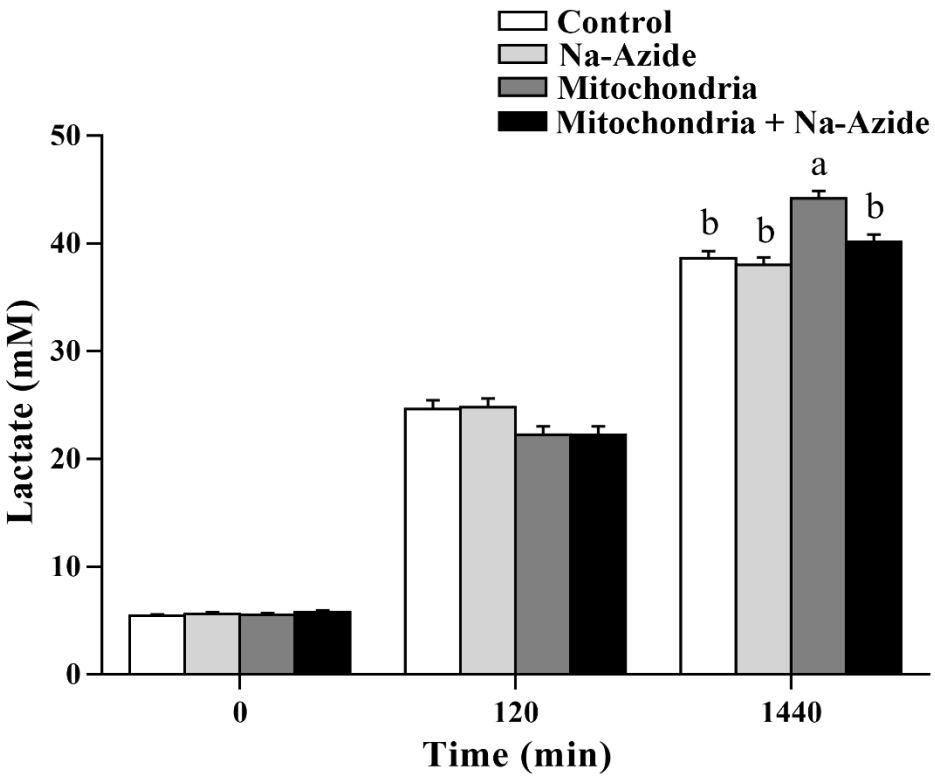

Fig. 4-3. Mean lactate (mM) of the *in vitro* model. Data are LS means ± SE. a,b means lacking a common letter differ within a time point ($P < 0.05$).

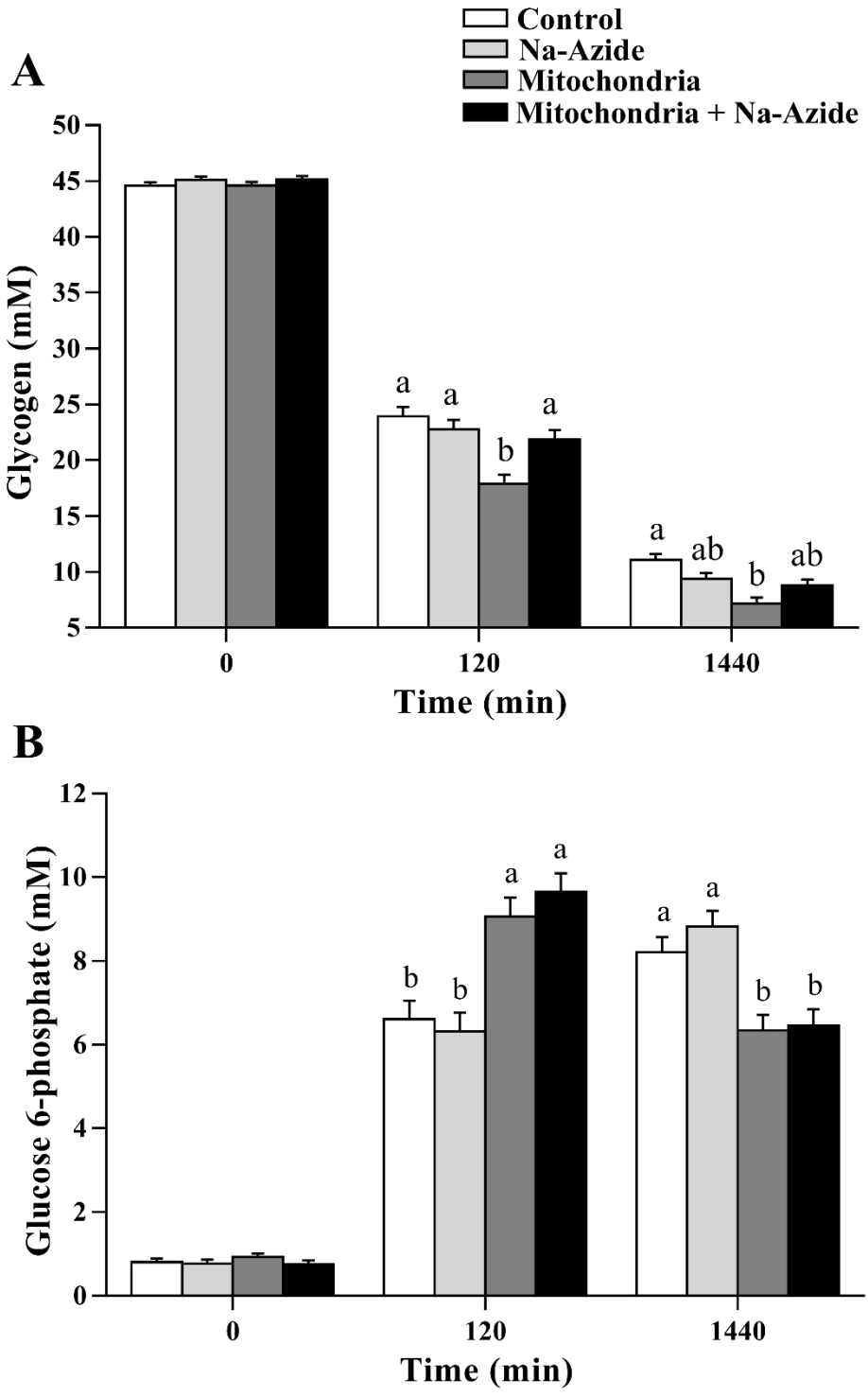

Fig. 4-4. Mean glycogen (mM; A), glucose 6-phosphate (mM; B) of the *in vitro* model. Data are LS means ± SE. a,b means lacking a common letter differ within a time point ($P < 0.05$).

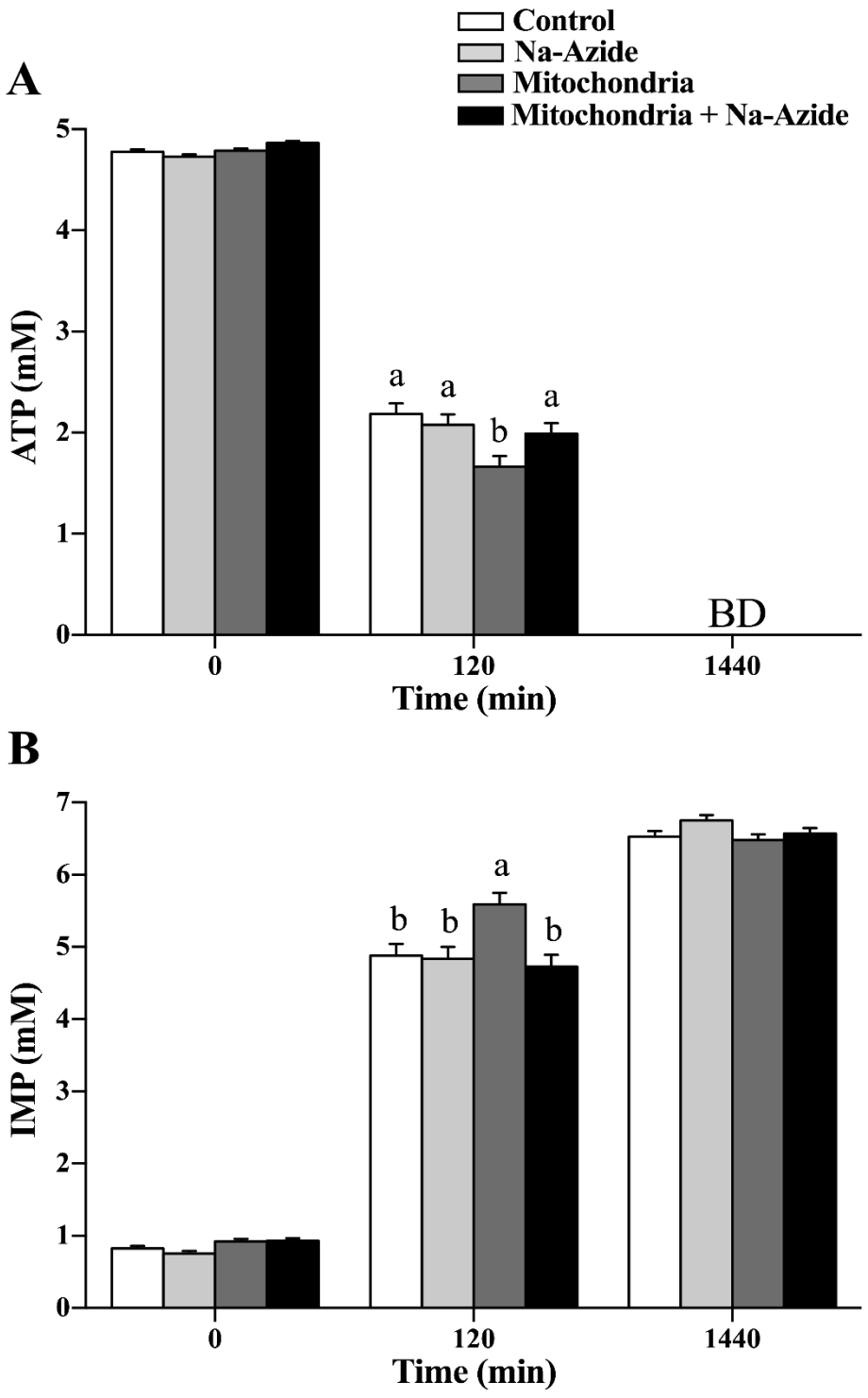

Fig. 4-5. Mean ATP (mM; A), IMP (mM; B) of the *in vitro* model. Data are LS means ± SE. a,b means lacking a common letter differ within a time point ($P < 0.05$). BD = below limit of detection.

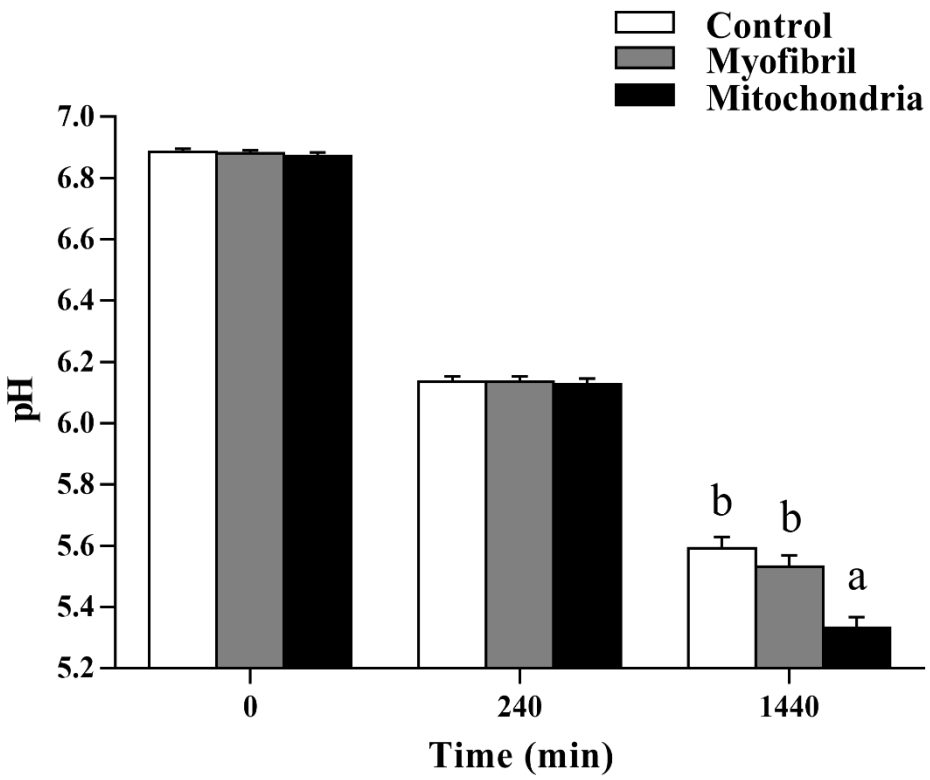

Fig. 4-6. Mean pH of the *in vitro* model. Data are LS means ± SE. a,b,c means lacking a common letter differ within a time point ($P < 0.05$).

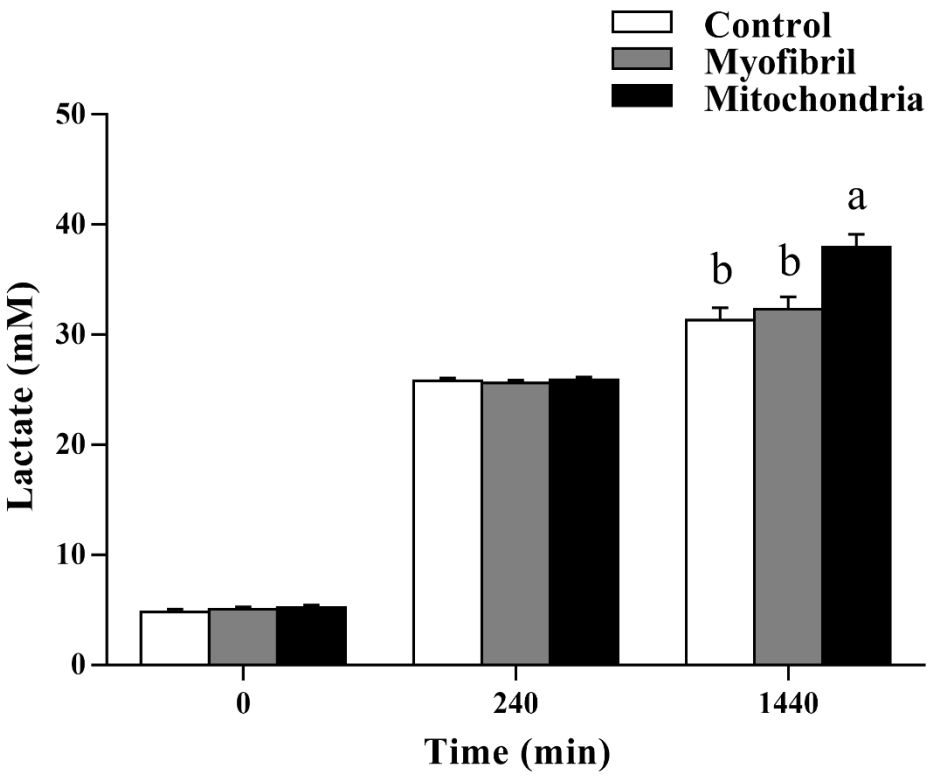

Fig. 4-7. Mean lactate (mM) of the *in vitro* model. Data are LS means ± SE. a,b,c means lacking a common letter differ within a time point ($P < 0.05$).

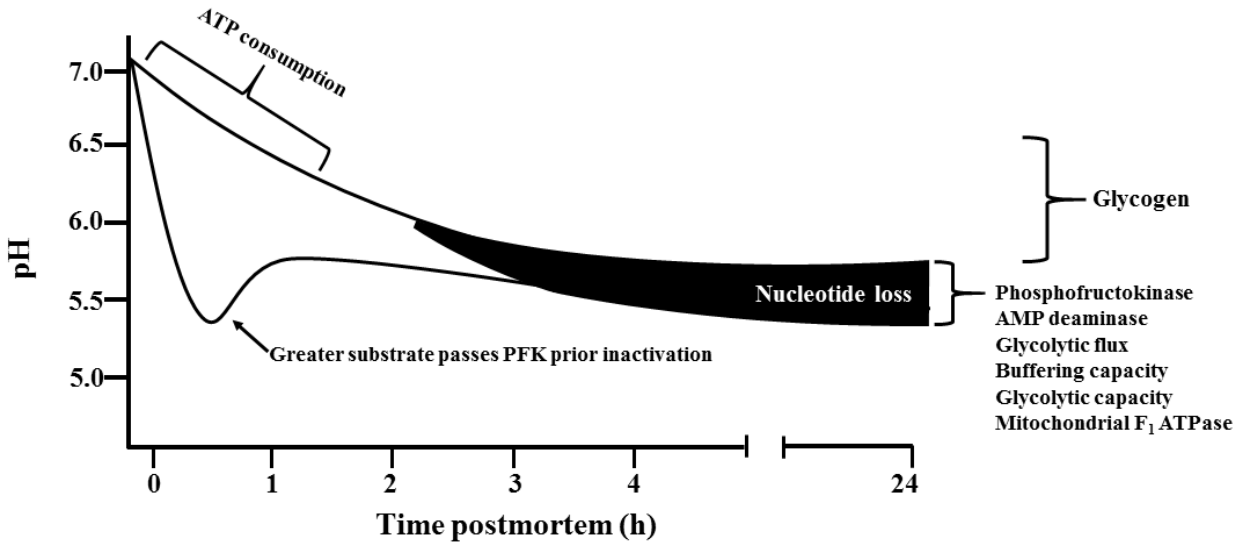

Fig. 4-8. Working model of the factor controlling the rate and extent of postmortem metabolism.

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Chapter 5 – Phosphofructokinase-1 and mitochondria partially explain the high ultimate pH of broiler *pectoralis major* muscle

Abstract

During postmortem metabolism muscle pH gradually declines to reach an ultimate pH near 5.6 across most meat species. Yet, broiler *pectoralis major* (*P. major*) muscle generates meat with high ultimate pH (pH ~ 5.9). For better understanding of the underlying mechanism responsible for this phenomenon, we evaluated the involvement of breast muscle chilling on the extent of postmortem metabolism. Broiler breast muscles were either subjected to chilling treatment (control) or left at room temperature (RT) for 120 min. *P. major* muscle from the RT treatment had lower ultimate pH, greater glycogen degradation and lactate accumulation. While these findings suggest that carcass chilling can contribute to the premature termination of postmortem metabolism, chilling did not fully explain the high ultimate pH of *P. major* muscle. Our results also revealed that glucose 6-phosphate (G6P) was very low at 24 h, and therefore we hypothesized that G6P was limiting. To test this hypothesis, muscle samples from *P. major* and porcine *longissimus lumborum* (*LL*) muscle were homogenized into a reaction buffer that mimics postmortem glycolysis with or without 0.5 mg/ml isolated mitochondria. While samples containing porcine *LL* muscle reached the normal level of ultimate pH, *P. major* muscle samples reached a value similar to that observed *in vivo* even in the presence of excess G6P, indicating that G6P was not limiting. Mitochondria enhanced the glycolytic flux and pH decline in system containing muscle from both species. More importantly, however, was that *in vitro* system containing chicken with mitochondria reached pH value similar to that of samples containing *LL* muscle without mitochondria. To investigate further, phosphofructokinase-1 (PFK-1) activity was compared in broiler *P. major* and porcine *LL* muscle at different pH values. PFK-1 activity

was lower in *P. major* muscle at pH 7, 6.5, and 6.2 than *LL* muscle. In conclusion, carcass chilling can partially contribute to the high ultimate pH of broiler *P. major* muscle, while low PFK-1 activity and mitochondria content limit the flux through glycolysis.

Key words: broiler breast, chilling, ultimate pH, phosphofructokinase-1, mitochondria

Introduction

Fresh meat quality is largely predicated on events occurring in muscle during its conversion to meat. While a number of production factors impact this process, the central dogma surrounding this crucial event is that anaerobic glycogen metabolism leads to the accumulation of lactate and hydrogen ions ($\mathbf{H^+}$) causing the pH of the tissue to fall (Bendall, 1973; Hamm, 1977). Under normal circumstances, muscle pH gradually drops from 7.2 at harvest to an ultimate pH around 5.5-5.7 and meat within this pH range exhibits the most desirable quality attributes (Van Laack et al., 2001). Yet, postmortem pH decline can stop prematurely resulting in a higher ultimate pH (pH > 5.8), which can lead to dark, firm, and dry (**DFD**) meat condition (Page et al., 2001; Viljoen et al., 2002). While this condition is predominantly observed in beef cattle, it also occurs in other meat species (Warriss et al., 1984, 1989; Allen et al., 1997). In broiler chicken, breast muscle usually exhibits an elevated ultimate pH (pH > 5.9) (Qiao et al., 2001; Alvarado and Sams, 2002; Souza et al., 2005; Zhu et al., 2013), however, DFD does not appear to be a problem for the poultry industry (Qiao et al., 2001; Lesiów and Kijowski, 2003). This is likely due to the low concentration of myoglobin in broiler breast muscle (Nishida and Nishida, 1985; Boulianne and King, 1995), which may prevent meat color darkening.

The premature cessation of postmortem metabolism is usually attributed the depletion of muscle glycogen as a result of prolonged antemortem stress. This is true if glycogen concentration at the time of death is less than 53 µmol/g of tissue (Henckel et al., 2002), but when concentrations are above this threshold, ultimate pH is determined by other factors. In the presence of residual glycogen, ultimate pH of meat is determined by the activity of the key regulatory enzyme phosphofructokinase-1 (**PFK-1**). We previously showed that PFK-1 starts to lose activity near pH 5.9 and becomes completely inactive at pH 5.5 which halts glycolytic flux and pH decline (England et al., 2014). In some cases, however, the depletion of adenine nucleotides (ATP, ADP, and AMP) arrest glycolysis while PFK-1 presumably is still functioning (England et al., 2016). Thus, any one or a combination of the aforementioned mechanisms can lead to the cessation of postmortem metabolism. More recently, we have reported that mitochondria can extend postmortem metabolism by increasing the flux through glycolysis *in vitro* (Matarneh et al., 2017), suggesting that the variations in the extent of postmortem metabolism may be more thoroughly explained by the abundance of mitochondria.

Carcass chilling is a crucial step in poultry processing to ensure high quality and safe meat product. Rapid carcass chilling markedly slows the rate of postmortem glycolysis and pH decline (Bowker et al., 2000; Stringer and Dennis, 2000). This is important for the poultry industry as rapid pH decline is the immediate reason for pale, soft, and exudative (**PSE**) meat defect (Rathgeber et al., 1999; Zhu et al., 2013). England et al. (2014) suggested that hastened glycolysis can extend postmortem pH decline through increasing the flux through glycolysis. Therefore, the opposite may also be true. To that end, we hypothesized that rapid carcass chilling reduces the flux through glycolysis, thereby causing premature termination of postmortem metabolism.

Materials and methods

Bird Slaughter and Muscle Sampling

All experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee at Virginia Tech. A total of 30 broiler chickens (mixed sex, 42 days of age, 3.0 ± 0.04 kg body weight) were obtained from the Virginia Tech Poultry Research Facility. Following a 10 h feed withdrawal period, all birds were harvested at the same facility using standard commercial procedures. Birds were stunned with carbon dioxide and immediately exsanguinated by severing both carotid arteries and at least one jugular vein. After bleeding for 120 s, skin was removed from the cranial part of the right *pectoralis major* (***P. major***) muscle and approximately 2 cm^3 sample was collected (subsequently referred to as 0 min sample; Figure 5-1). Samples were immediately snap frozen in liquid nitrogen, and stored at − 80 °C. Following, birds were scalded at 60 °C for 90 s, defeathered in a rotary drum picker for 30 s, and manually eviscerated. Immediately after evisceration, whole breast muscles were removed from each carcass, labeled, and assigned to one of two chilling treatments (n = 15 per treatment). Treatments were: chilling in ice water at 0.5 °C for 120 min (Control) or held at room temperature (**RT**) for 120 min. Following, muscles from both treatments were placed in cold storage room at 4 °C until 24 h postmortem. Additional muscle samples were collected and stored in the same manner as described for the 0 min samples at 30, 120, and 1440 min (24 h) postmortem from the right *P. major* muscle (Figure 5-1).

Temperature

Muscle internal temperature was measured at 10, 30, 60, 120, 240, and 1440 min postmortem through an incision made by a knife in the cranial part of the left *P. major* muscle (Figure 5-1) using a data logger thermometer (HH147U; Omega Engineering, Inc., Norwalk, CT, USA).

Pectoralis major muscle pH and metabolite analysis

Frozen 0, 30, 120, 1440 min *P. major* muscle samples were powdered under liquid nitrogen using a mortar and pestle, and three tubes of approximately 0.1 g were collected. For pH analysis, powdered muscle samples were lysed using a Tissue Lyser II system (Qiagen, Boston, MA, USA) in 0.8 ml of ice-cold solution containing 5 mM sodium iodoacetate and 150 mM KCl (pH 7.0) (Bendall, 1973). Samples were then centrifuged at 13,000 rpm for 5 min, equilibrated to 25 °C, and measured directly using an Orion Ross Ultra pH glass electrode (Thermo Scientific, Pittsburgh, PA, USA). Samples designated for glucose, glucose-6-phosphate (**G6P**), lactate, adenine nucleotides, and inosine monophosphate (**IMP**) analysis were lysed in 1 ml of ice-cold 0.5 M perchloric acid. After incubating on ice for 20 min, homogenates were centrifuged at 13,000 rpm for 5 min, and the resulting supernatants were transferred to new tubes and neutralized with 2 M KOH (Bergmeyer, 1984). For muscle glycogen determination, another sample was lysed in 1 ml of 1.25 M HCl, heated at 90 °C for 2 h, and centrifuged at 13,000 rpm for 5 min. Supernatants were transferred to new tubes and neutralized with 1.25 M KOH (Bergmeyer, 1984). Glycogen, glucose, G6P, and lactate were determined using enzymatic methods modified for a 96-well plate as described by (Hammelman et al., 2003). Adenine nucleotide and IMP contents were quantified using HP Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) and external standards (Bernocchi et al., 1994; Williams et al., 2008)

Porcine muscle sampling

Market-weight pigs (n = 6) were slaughtered in the Virginia Tech Meat Center using standard commercial procedures. Muscle samples were excised from the *longissimus lumborum* (***LL***)

muscle at 0 min (within 5 min of exsanguination) postmortem. Samples were used for mitochondrial extraction or immediately snap frozen in liquid nitrogen, and stored at − 80 °C.

Mitochondria isolation

Mitochondria were isolated from porcine *LL* muscle by differential centrifugation according to (Scheffler et al., 2015). Briefly, muscle samples were finely minced with scissors in ice-cold isolation buffer (5 ml/g of muscle; 100 mM sucrose, 180 mM KCl, 50 mM Tris, 5 mM $MgCl_2$, 10 mM EDTA, 1 mM K-ATP, pH 7.4). Protease (subtilisin A) was added to the tissue suspension at 0.4 mg/ml followed by homogenization with a Potter-Elvehjem type homogenizer system (Glas-Col, Terre Haute, IN. USA). Homogenates were diluted with isolation buffer to achieve ~ 20 ml/g of muscle before filtering through two layers of cheese-cloth. Homogenates were then centrifuged at 1,000 × g for 10 min at 4 °C followed by a second filtration of the supernatant through cheese-cloth. Filtered supernatants were centrifuged again at 8,000 × g for 10 min at 4 °C. Resulting mitochondrial pellets were suspended in mitochondrial suspension buffer (220 mM mannitol, 70 mM sucrose, 10 mM Tris–HCl, and 1 mM EGTA, pH 7.4). Mitochondrial protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL. USA).

In vitro glycolysis model

Frozen 0 min *P. major* (n = 6) and porcine *longissimus lumborum* (n = 6) muscles were pulverized under liquid nitrogen and homogenized at 1:10 (wt/vol) in reaction buffer containing 40 mM glycogen, 60 mM KCl, 5 mM $MgCl_2$, 10 mM Na_2HPO_4, 30 mM creatine, 25 mM carnosine, 10 mM sodium acetate, 5 mM ATP, 0.5 mM ADP, and 0.5 mM NAD^+ (pH 7.4) (England et al., 2014). Either 0 or 0.5 mg/ml isolated mitochondria were incorporated into the *in vitro* model. Aliquots were removed at 0, 30, 120, 240, and 1440 min for pH and metabolite analysis. Reaction vessels were maintained at 25 °C for the duration of the trial.

In vitro pH and metabolite analysis

Aliquots for pH analysis were mixed with 25 mM sodium iodoacetate and 750 mM KCl solution (pH 7.0) at 4:1 (vol/vol). Samples were then centrifuged at 13,000 rpm for 5 min at room temperature, equilibrated to 25 °C, and measured directly using an Orion Ross Ultra pH glass electrode (Thermo Scientific, Pittsburgh, PA, USA). Samples for glucose, G6P, and lactate determination were added to ice-cold 1 M perchloric acid at 1:1 ration. After incubating on ice for 20 min, samples were centrifuged at 13,000 rpm for 5 min, and the resulting supernatants were neutralized with 2 M KOH. Aliquots for glycogen analysis were mixed with equal volume of 2.5 M HCl, heated at 90 °C for 2 h, centrifuged at 13,000 rpm for 5 min. Supernatants were transferred to new tubes and neutralized with 1.25 M KOH. Glycogen, glucose, G6P, and lactate were measured according to (Hammelman et al., 2003).

Phosphofructokinase-1 activity assay

Phosphofructokinase-1 activity of *P. major* (n = 5) and porcine *LL* (n = 5) muscles was determined according to the procedures outlined by (England et al., 2014). Briefly, ~ 0.1 g of the 0 min samples was homogenized at 1:10 (wt/vol) in 100 mM K_2HPO_4 solution (pH 7.4). Aliquots of tissue homogenate were added to a reaction buffer containing 120 mM MES, 3.2 mM $MgSO_4$, 2 mM ATP, 1 mM NADH, 3 mM fructose 6-phosphate, 2 U/mL triosephosphate isomerase, 1 U/mL glycerol-3-phosphate dehydrogenase and 1 U/mL aldolase. The pH of the buffer was adjusted to 7.0, 6.5, 6.2, 6.0, and 5.8. Assays were carried out at 25 °C and the reduction in absorbance due to the oxidation of NADH to NAD^+ was measured spectrophotometrically at 339 nm. Maximum PFK-1 activity was calculated and reported as nmol NADH $*$ min^{-1} $*$ mg^{-1}.

Statistical analysis

Effects of chilling treatment and time on metabolites and pH were analyzed as a split-plot design using the mixed model of JMP (SAS institute Inc., Cary, NC). The statistical model included the main effects of chilling treatment and time and their interaction, with *P. major* muscles as main plots and times as subplots. For the *in vitro* study, data were also analyzed with a split-plot design. The statistical model included main effects of species (chicken or pork), mitochondria (0 or 0.5), time, and their interactions. The *in vitro* models (tubes) were considered as the main plots and times as subplots. The slice function was used to determine treatment effects at individual time points. Data determining PFK-1 activity between species were compared within each pH value. Means were evaluated using a Student's t-test and considered significant at $P \leq 0.05$. All data are expressed as least-squares means ± SE.

Results and discussion

Temperature and pH

In our initial experiment, we studied the impact of breast muscle chilling on postmortem glycolysis and pH decline. The difference in temperature declines between breast muscles immediately immersion chilled (control) and muscles held at room temperature (RT) for 2 h is readily apparent in Figure 5-2. A significant treatment × time interaction ($P < 0.0001$) was observed for *P. major* muscle temperature. At 10 min postmortem (pre-chill), there was no difference in breast muscle temperature among treatments with a mean of 40.5 ± 0.1 °C. As expected, at 30, 60, 120, and 240 min postmortem, higher mean temperature ($P < 0.0001$) was observed in muscles from the RT treatment compared to control. These results affirm that our chilling treatment produced differing cooling rates in the *P. major* muscle.

The chilling treatment significantly influenced pH decline of the *P. major* muscle (P = 0.02; Figure 5-3). While no differences were detected through 120 min postmortem, a lower ultimate pH (at 1440 min, P = 0.02) was observed in muscles from the RT treatment (5.8 ± 0.01) compared to control (5.9 ± 0.01). These findings are in agreement with previously published reports (McKee and Sams, 1998; Alvarado and Sams, 2002; Öztürk and Serdaroglu, 2015), where higher carcass temperatures were associated with lower ultimate pH. However, breast muscle from the RT treatment still possesses a greater than normal ultimate pH, suggesting other mechanisms may be involved in determining the extent of postmortem pH decline of broiler breast muscle.

Glycolytic metabolites and adenine nucleotides

For better understanding of postmortem metabolism, glycolytic metabolite and adenine nucleotide levels were measured in the *P. major* muscle. During the postmortem period, pyruvate generated through glycolysis is converted to lactate and accumulates in the muscle. As expected, patterns of lactate formation in the current study followed pH decline. Lactate concentration of the *P. major* muscle was not affected by the chilling treatment through 120 min. However, at 1440 min, muscles from the RT treatment had significantly greater lactate (P = 0.01; Figure 5-4A) when compared to that of control. These results confirm that lower ultimate pH observed in the RT treatment was due to greater flux through the glycolytic pathway. It is well established that rapid cooling of carcasses slows metabolic enzyme activity and limits rate of metabolism (Bowker et al., 2000). Bock and Frieden (1974) reported that PFK-1 loses as much as 97% of its activity as temperature reduces from 20 °C to 3 °C. The authors suggested that lower temperature enhances enzyme dissociation from its more active tetrameric form to a less active

dimeric form. This in turn reduces glycolytic flux, resulting in high ultimate pH (England et al., 2014).

Mobilization of muscle glycogen during postmortem metabolism drives pH decline. Therefore, inadequate muscle glycogen can lead to premature cessation of postmortem metabolism (Henckel et al., 2002). Glycogen degradation yields glucose 1-phospahte (**G1P**) and non-phosphorylated glucose molecules. Subsequently, G1P is isomerized by phosphoglucomutase to G6P and enters the glycolytic pathway, while free glucose accumulates in postmortem muscle (Young et al., 1988; Scheffler and Gerrard, 2007). At 30 min postmortem, glycogen was lower in samples from the RT treatment compared to controls ($P = 0.04$; Figure 5-4B). However, no differences in glycogen among treatments were observed at 120 and 1440 min. The overall mean glycogen concentration at 0 min was 37 ± 1.5 µmol/g of muscle; a concentration lower than those found in *LL* muscle of pork (Copenhafer et al., 2006; Matarneh et al., 2015) and beef (Frylinck et al., 2013; Apaoblaza et al., 2015). The low glycogen concentration in *P. muscle* of current commercial broilers has been attributed to the intensive selection for increased breast yield (Berri et al., 2005; Le Bihan-Duval et al., 2008). Even so, however, glycogen was not depleted in either treatment at 1440 min, indicating that glycogen was not limiting and postmortem glycolysis could have continued. The impact of chilling treatment on G6P was dependent on time (treatment × time, $P = 0.03$, Figure 5-4C). While no difference was observed at 30 min postmortem, lower G6P ($P = 0.0004$) was detected at 120 min in muscles from the RT treatment compared to control. Glucose concentration was also significantly affected by the chilling treatment over time (treatment × time, $P = 0.0002$; Figure 5-4D). Breast muscles left at room temperature had lower glucose levels at 120 and 1440 min ($P \leq 0.002$) compared to those from the control treatment. When glycogen is not a limiting factor,

G6P accumulates in muscle to reach a concentration of about 10 µmol/g by 24 h postmortem across most meat species (Copenhafer et al., 2006; Apaoblaza et al., 2015; Matarneh et al., 2015). Surprisingly, at 1440 min, G6P was very low regardless of the chilling treatment. This suggests that glycogen degradation was either greatly repressed or G6P was generated at a rate comparable to that of consumption.

The rate of postmortem glycolysis is directly related to the rate of ATP hydrolysis (Scopes, 1974). Mean muscle ATP concentration of the RT treatment was lower than control at 30 min postmortem (P = 0.02; Figure 5-5A). At 120 min, no difference was found among treatments, while at 1440 min ATP was below the limits of detection in both treatments. ATP splitting rate by muscle ATPases decreases with decreasing temperature from 38 to 15 °C (Newbold and Scopes, 1967), which explains the greater ATP concentration at 30 min in control treatment. At 30 min postmortem, *P. major* muscles from the RT treatment had significantly lower ADP content (P = 0.03; Figure 5-5B) when compared to control. At 120 and 1440 min, there were no significant differences in ADP among treatments. AMP was below the limits of detection at 30 min postmortem. However, a trend toward greater AMP values were observed in breast muscles from the control treatment at 120 min in comparison to those of the RT (P = 0.08, Figure 5-5C). Greater IMP concentration (P = 0.04; Figure 5-5D) was detected at 30 min in muscles from the RT treatment than control. However, differences in IMP among treatments was not observed at 120 and 1440 min. As ADP concentration rises due to ATP hydrolysis in postmortem muscle, adenylate kinase catalyzes the dismutation reaction between two molecules of ADP to generate ATP and AMP. Once formed, AMP is converted to IMP by AMP deaminase, which leads eventually to the depletion of the adenine nucleotide pool (Greaser, 1986). The loss of adenine nucleotides has previously been shown to mediate premature termination of

postmortem metabolism (England et al., 2016). Yet, adenine nucleotides were not depleted by 120 min, suggesting that adenine nucleotides were not limiting, at least during the first 120 min postmortem. Combined with pH and metabolite data, our findings suggest that breast muscle chilling only partially explain the high ultimate pH in broiler *P. major* muscle. Further, postmortem glycolysis was terminated in the presence of residual glycogen which eliminates glycogen from being the causative agent. On the other hand, however, the low G6P concentration at 1440 min raises the possibility that glycogenolysis was inhibited, which makes G6P potentially responsible for the termination of postmortem glycolysis.

In vitro pH and metabolite

To further test the aforementioned, we utilized an *in vitro* system designed to recapitulate muscle postmortem glycolysis in the presence of excess glycogen. The *in vitro* system composed of a buffer containing all metabolites required for glycolysis in addition to muscle tissue to serve as the source of glycolytic enzymes. Using this *in vitro* system, we were able to compare glycolysis and pH decline in broiler *P. major* and porcine *LL* muscles with or without 0.5 mg/ml isolated mitochondria under the same environment. Because the *in vitro* system was originally used with porcine *LL* muscle (England et al., 2014, 2015; Scheffler et al., 2015), the same muscle was used in the current study as a positive control. Finally, mitochondria were incorporated because our recently published research indicated that mitochondria can extended postmortem glycolysis in an *in vitro* system containing porcine *LL* muscle (Matarneh et al., 2017). Therefore, mitochondria were used to test whether the same effect could be generated in system containing broiler *P. major* muscle.

The pH of the *in vitro* system was significantly affected by the interaction between species and time ($P < 0.0001$) and mitochondria and time ($P < 0.0001$) (Figure 5-6A). At 120

min, mitochondria significantly lowered the pH in system containing *P. major* muscle (P = 0.0001), and in both muscles at 240 and 1440 min ($P < 0.0001$) compared to those containing the same muscle without mitochondria. Reaction vessels containing *P. major* regardless of mitochondria had lower pH at 120 min ($P < 0.0001$) in comparison to their *LL* counterparts. At 1440 min, reaction containing *LL* muscle with mitochondria had the lowest ($P < 0.0001$) pH value (5.39 ± 0.06), while reactions containing *P. major* without mitochondria had the highest value (5.96 ± 0.04). Further, no difference in ultimate pH between *LL* without mitochondria and *P. major* with mitochondria was detected at 1440 min. At 120 min, samples containing *P. major* muscle regardless of mitochondria had greater lactate levels compared to those containing *LL* muscle (P = 0.0008; Figure 5-6B). At 240 min, mitochondria contributed to greater lactate accumulation in system containing *P. major* but not in the ones containing *LL* muscle (P = 0.001). Reactions containing *LL* muscle with mitochondria had the greatest lactate concentration at 1440 min, while *P. major* without mitochondria had the lowest ($P < 0.0001$). Regardless of mitochondria, enhanced glycogen degradation was observed in system containing *P. major* muscle compared to those containing *LL* muscle from 120 to 1440 min ($P \leq 0.04$; Figure 5-7A). Within the same species, mitochondria lowered glycogen concentration in *P. major* samples at 120 and 240 min and in samples from both species at 1440 min ($P \leq 0.02$). Samples containing *P. major* muscle with mitochondria had the lowest glycogen levels from 120 to 1440 min, while samples containing *LL* without mitochondria had the greatest levels at the same time points ($P \leq$ 0.02). Reaction vessels containing *LL* with mitochondria had the greatest G6P concentration at 30 min, while the lowest concentration was observed in *P. major* without mitochondria samples ($P < 0.0001$; Figure 5-7B). In the presence of mitochondria, greater G6P concentration was

detected at 120 min in both muscles ($P < 0.0001$). At 240 and 1440 min, *P. major* muscle regardless of mitochondria had greater G6P than those containing *LL* muscle ($P \leq 0.0002$).

Our *in vitro* data showed that the pH decline of samples containing *P. major* muscle without mitochondria arrested prematurely, resulting in a high ultimate pH which was comparable to those measured *in vivo* (Figure 5-3). In contrast, the ultimate pH of system containing porcine *LL* muscle was about 0.3 pH units lower than that of *P. major* treatment (Figure 5-6A). The lower ultimate pH in *LL* muscle treatment was associated with greater flux through the glycolytic pathway, as evidenced by greater lactate accumulation at 1440 min (Figure 5-6B). Because muscle samples from both species were homogenized in the exact same buffer, differences in ultimate pH should be a function of the incorporated muscle tissue.

Contrary to *in vivo* findings, G6P accumulated in samples containing *P. major* muscle to reach about 10 mM after 1440 min, (Figure 5-7B) which argues against G6P being the reason for the cessation of postmortem metabolism in *P. major* muscle. The termination of postmortem metabolism in the presences of residual glycogen and glycolytic metabolites is a function of adenine nucleotides depletion or pH-mediated inactivation of PFK-1 (Kastenschmidt et al., 1968; Greaser, 1986; England et al., 2014, 2016). To test whether adenine nucleotides disappearance is the reason for the cessation of postmortem metabolism in *P. major* muscle, we added 24 h *P. major* muscle to the *in vitro* system with 3 mM ATP (pH 5.9). We postulated that if adenine nucleotides were limiting, the addition of ATP would drive additional pH decline. Yet, further pH decline or lactate accumulation was not observed (data not shown), suggesting that adenine nucleotides were not limiting. Correspondingly, these data indicate that pH inactivation of PFK-1 is likely the culprit, a notion supported by the accumulation of G6P in the *in vitro* system. Once

inactivated, flux through PFK-1 is halted leading to the accumulation of G6P and fructose 6-phosphate in postmortem muscle.

We have recently shown that mitochondria can extend pH decline though increasing the flux through glycolysis *in vitro* (Matarneh et al., 2017). Similarly, mitochondria promoted glycogen degradation, G6P and lactate accumulation, and pH decline in the present study. While the exact mechanism by which mitochondria extend pH fall is still unclear, we have recently shown that mitochondria can accelerate glycolysis as mitochondrial F_1F_0 ATP synthase operates reversely and hydrolyzes ATP (Matarneh, 2017). Hastened glycolysis allows more substrate to pass PFK-1 prior to inactivation, thereby extends pH decline (England et al., 2014). Additionally, mitochondria may increase the NAD^+/NADH ratio in the cytosol (Jong and Davis, 1983), allowing greater flux through glycolysis. Curiously, samples containing *P. major* muscle with mitochondria had similar pH value to that of *LL* muscle without mitochondria. Therefore, we suggest that mitochondria may partially explain the lower ultimate pH in beef and pork *LL* muscle than chicken *P. major* (lower mitochondrial content). These data indicate that mitochondria can participate to postmortem metabolism and play a role in controlling ultimate pH. Furthermore, variations in the extent of postmortem pH decline may be more thoroughly explained and predicted by the abundance of mitochondria.

PFK-1 activity

In an attempt to further establish the role that PFK-1 plays in terminating pH decline in broiler *P. major* muscle, PFK-1 activity was compared between *P. major* and porcine *LL* muscle at pH 7, 6.5, 6.2, 6, and 5.8. PFK-1 activity was significantly affected by the interaction between species and pH ($P < 0.0001$; Figure 5-8). Enzyme activity was dramatically affected by the drop in pH ($P < 0.0001$). Our results show that PFK-1 loses as much as 65% of its activity as pH drops from

7 to 6.5. On the other hand, enzyme activity was about 100% greater in *LL* than *P. major* muscle at pH 7 and the difference was maintained at pH 6.5 and 6.2 ($P \leq 0.009$). Yet, the difference was lost at pH 6.0 and 5.8. These findings are consistent with those of (England et al., 2014) who found that PFK-1 activity was markedly impaired due to postmortem pH decline. High concentrations of H^+ promote the dissociation of the more active tetramer form into less active dimer form of the enzyme. Low PFK-1 activity may limit flux through glycolysis leading to premature termination of postmortem metabolism.

In conclusion, present work demonstrated that carcass chilling can partially contribute to the high ultimate pH of broiler *P. major* muscle. Our results also indicated that the cessation of postmortem metabolism in *P. major* muscle is not related to substrate availability (glycogen, G6P, or adenine nucleotides). Instead, broiler *P. major* muscle exhibits lower PFK-1 activity than porcine *LL* muscle, which may reduce the flux through glycolysis. Further, the inclusion of mitochondria to an *in vitro* model system containing *P. major* muscle enhance glycolytic flux and extended pH decline to a value similar to the normal ultimate pH.

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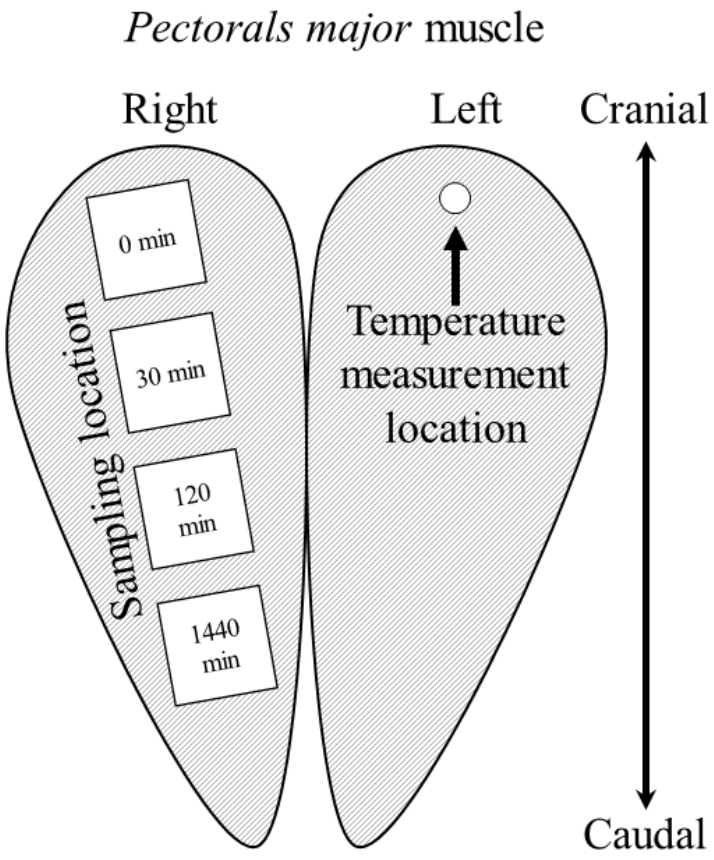

Figure 5-1. Schematic diagram of muscle sampling lactations and temperature measurements.

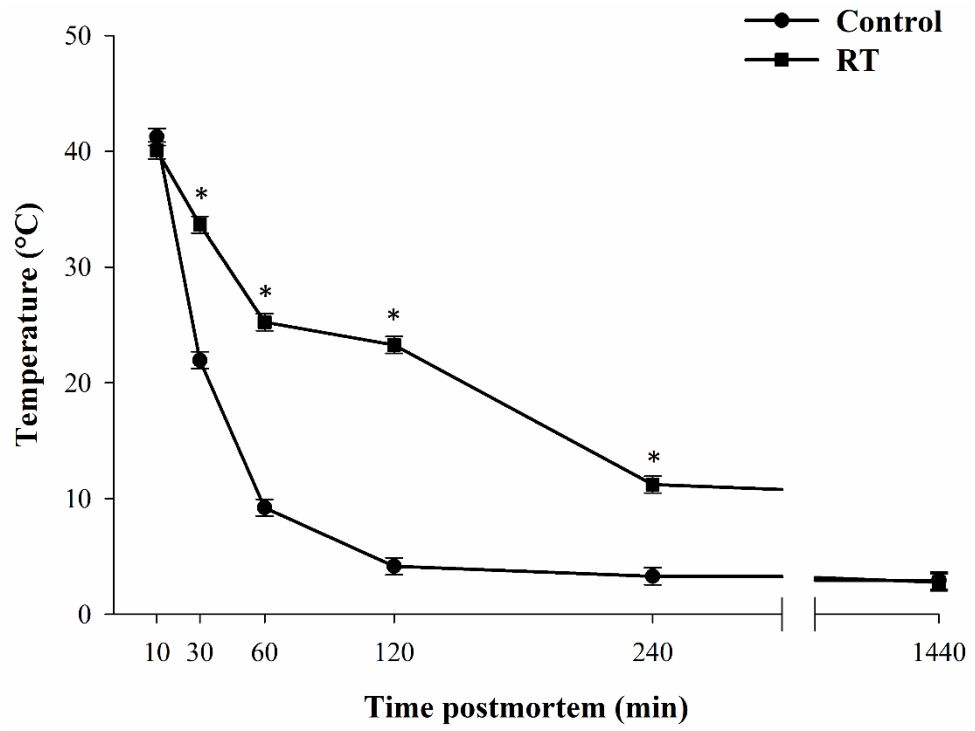

Figure 5-2. Broiler *P. major* muscle temperature (°C) of control and RT treatments. Data are LS means ± SE. *indicates significant difference within a time point ($P < 0.05$).

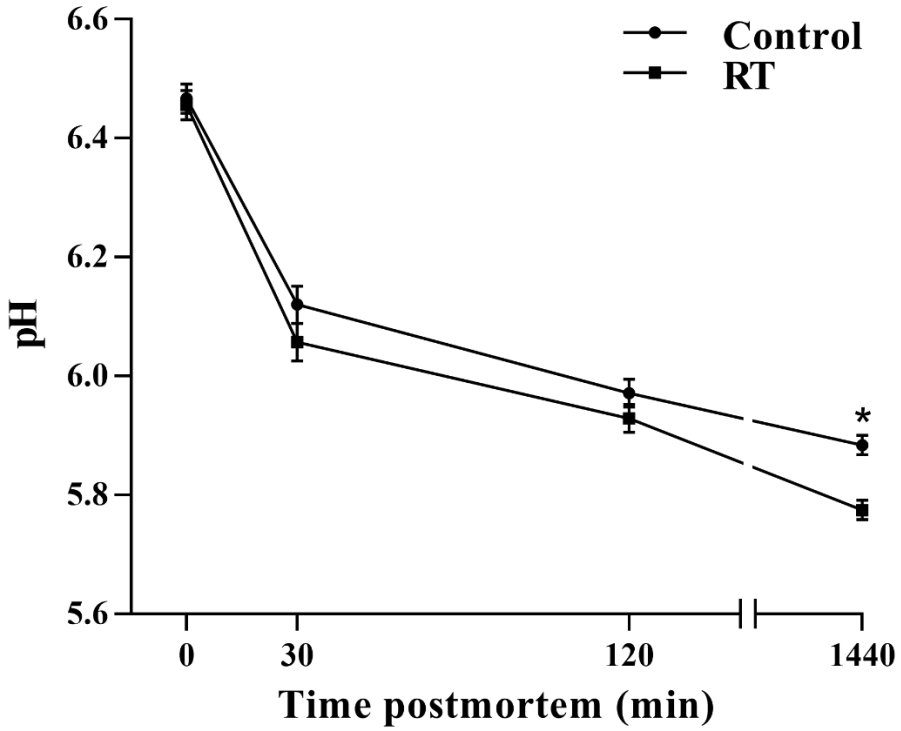

Figure 5-3. Mean pH in broiler *P. major* muscle of control and RT treatments. Data are LS means ± SE. *indicates significant difference within a time point ($P < 0.05$).

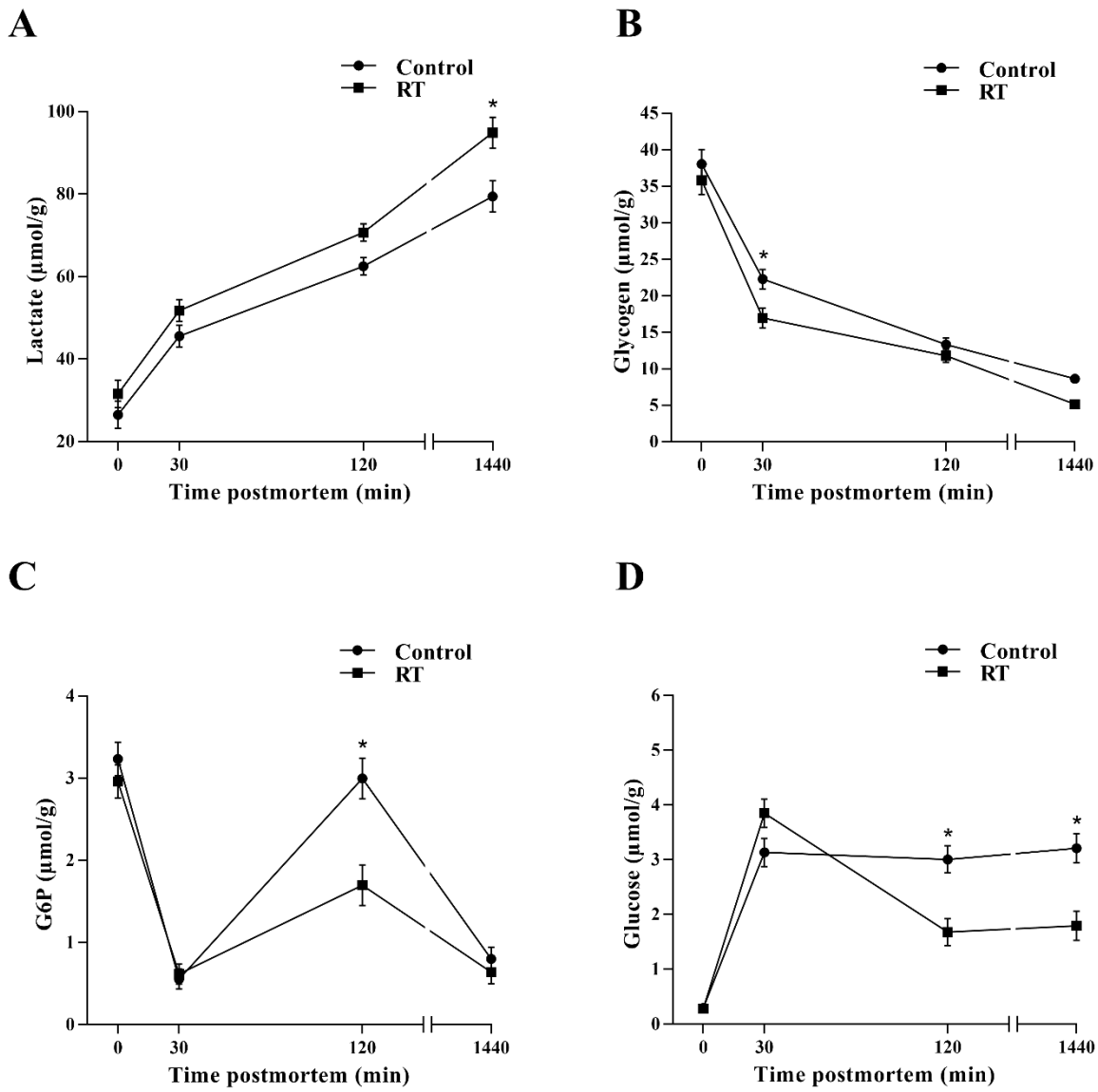

Figure 5-4. Mean lactate (A; µmol/g), glycogen (B, µmol/g), G6P (C; µmol/g) and glucose (D; µmol/g) in broiler *P. major* muscle of control and RT treatments. Data are LS means ± SE. *indicates significant difference within a time point ($P < 0.05$).

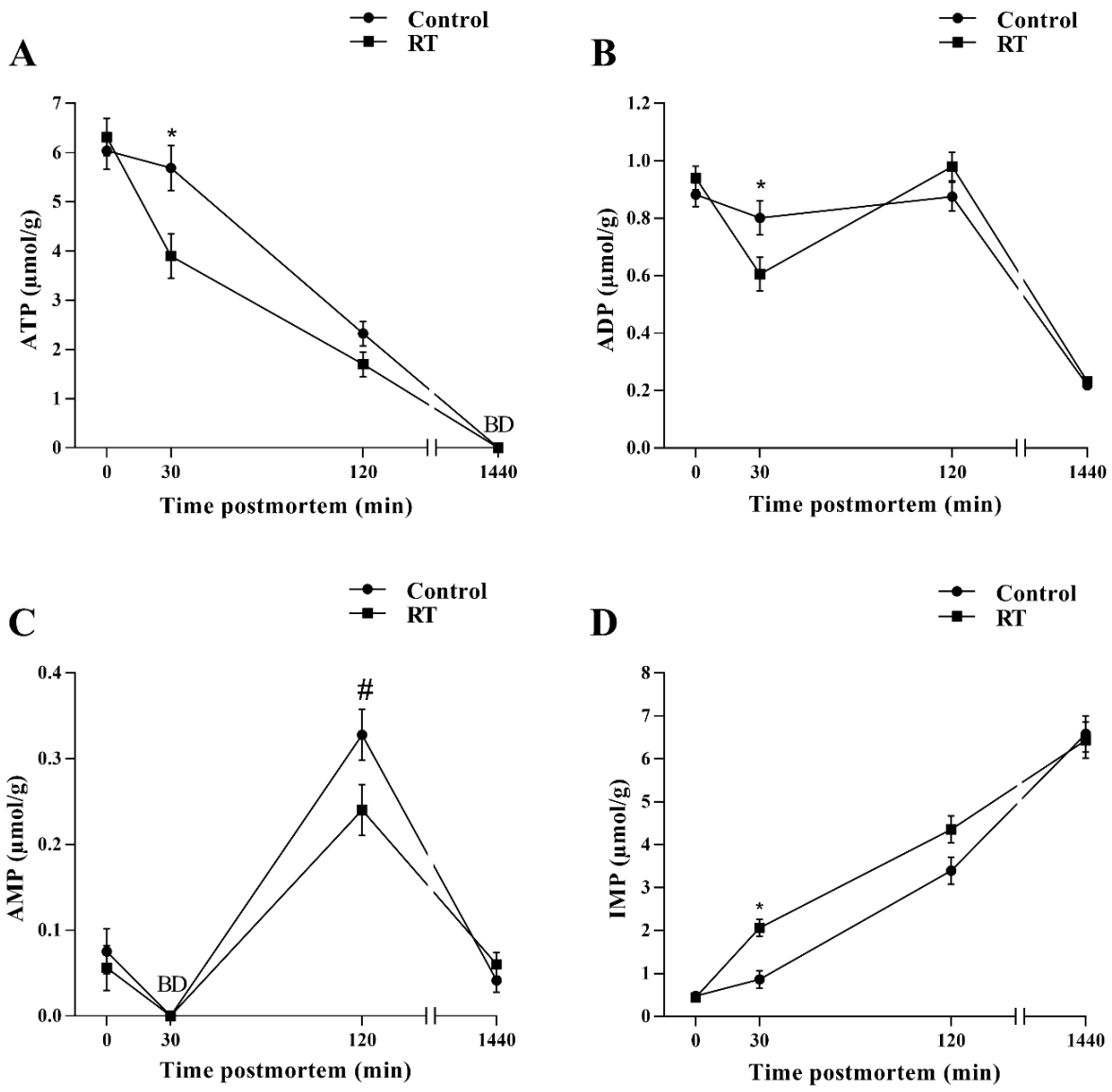

Figure 5-5. Mean ATP (A; µmol/g), ADP (B, µmol/g), AMP (C; µmol/g) and IMP (D; µmol/g) in broiler *P. major* muscle of control and RT treatments. Data are LS means ± SE. *indicates significant difference within a time point ($P < 0.05$). #indicates tendency for difference within a time point ($P < 0.1$). BD = below limit of detection.

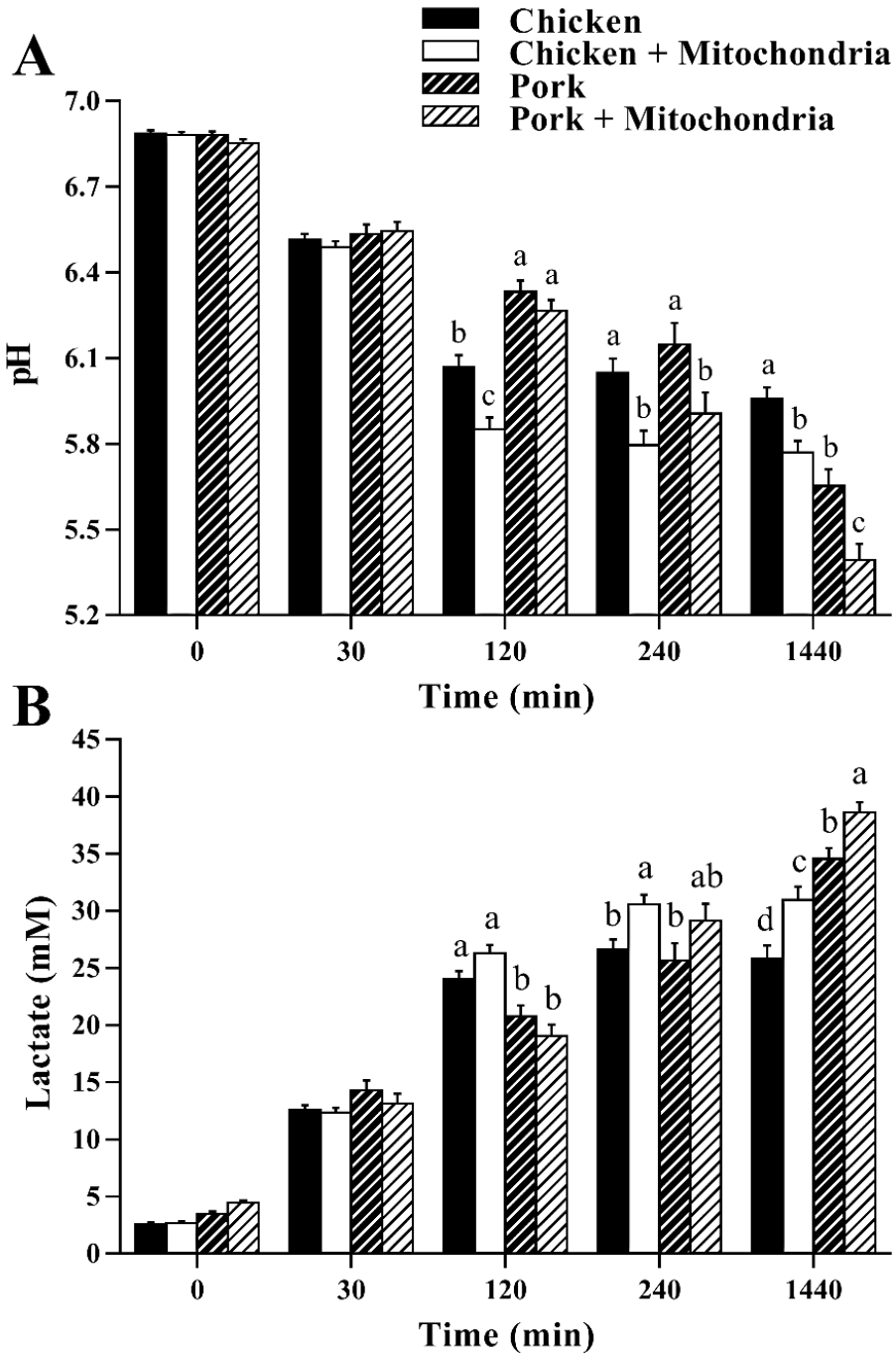

Figure 5-6. Mean pH (A) and lactate (B; mM) of the *in vitro* model. Data are LS means ± SE. a,b,c,d means lacking a common letter differ within a time point ($P < 0.05$).

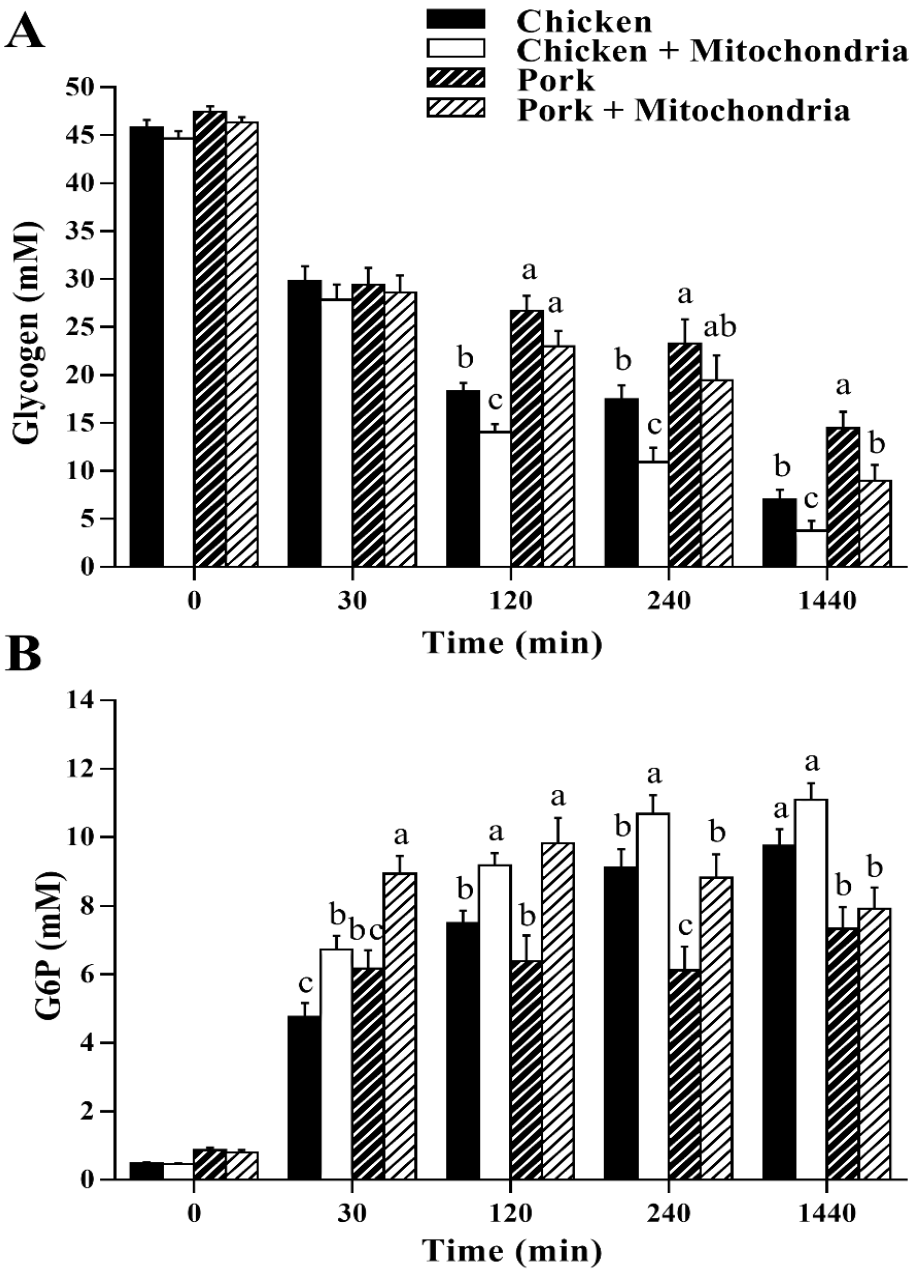

Figure 5-7. Mean glycogen (A; mM) and G6P (B; mM) of the *in vitro* model. Data are LS means ± SE. a,b,c means lacking a common letter differ within a time point ($P < 0.05$).

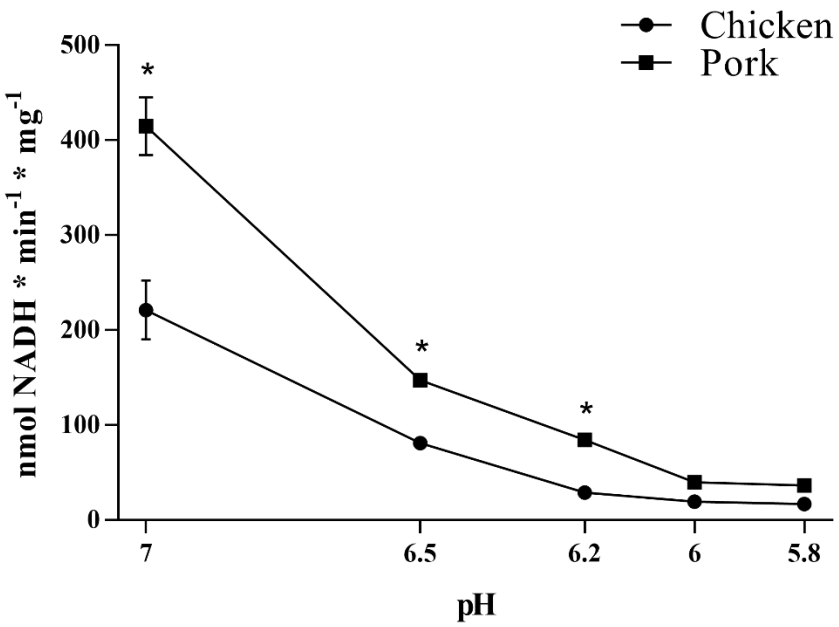

Figure 5-8. Phosphofructokinase-1 activity comparison between broiler *P. major* and porcine *LL* muscle at pH 7, 6.5, 6.2, 6, and 5.8. Data are LS means ± SE. *indicates significant difference within a pH value ($P < 0.05$).

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