Postmortem metabolism in porcine skeletal muscle

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Abstract

Once an animal is harvested for meat, skeletal muscle attempts to maintain ATP at or near antemortem levels. To maintain ATP levels postmortem, stored glycogen is catabolized to produce ATP through glycolysis and possibly oxidative metabolism. Hydrolysis of the produced ATP acidifies muscle until an ultimate pH is reached. The ultimate pH of meat directly impacts the quality characteristics of color, texture, and water holding capacity. Therefore, our research intends to describe the contributions glycolysis and oxidative metabolism play in determining ultimate pH and fresh meat quality. Traditionally, glycogen content at death was thought to be responsible for dictating ultimate pH. This was especially true in oxidative muscle with limited glycogen stores. Yet, our research indicated that in the presence of excess glycogen, oxidative muscle maintains a high ultimate pH. Rather, pH inactivation of phosphofructokinase is responsible for terminating postmortem glycolysis and brackets ultimate pH between 5.9 – 5.5. Meat with a pH below this range is uncommon. However, AMPK $\gamma3^{R200Q}$ mutant pigs produce meat with an ultimate pH near 5.3. Due to lower AMP deaminase abundance in their muscle, AMP levels are elevated late postmortem. Because AMP is a potent activator of phosphofructokinase, the aberrant meat quality from AMPK $\gamma3^{R200Q}$ mutant pigs is caused by extended postmortem glycolysis. Combined, these data further our understanding of the factors that contribute to the formation of fresh meat quality.

We also characterized AMPK $\gamma3^{R200Q}$ muscle by investigating antemortem skeletal muscle lactate transport. Lactate is transported in or out of tissues by proton-linked

monocarboxylate transporters (MCTs). Previous reports indicated that acute activation of AMPK increased monocarboxylate transporter expression in skeletal muscle of other species. Yet, it was unknown the impact chronic activation of AMPK will have on MCT1, MCT2, and MCT4 expression in pigs. Compared to wild-type pigs, the longissimus lumborum of AMPK $\gamma3^{R200Q}$ pigs increased both MCT2 and MCT4 protein expression. Our data suggest glycolytic skeletal muscle from the AMPK $\gamma3^{R200Q}$ pigs has increased capacity for antemortem lactate export from muscle and possibly increased pyruvate transport into the mitochondria.

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Chapter 1 – Exploring the unknowns involved in the transformation of muscle to meat

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Abstract

Meat quality development, or the transformation of muscle to meat, involves a myriad of biochemical pathways that are largely well-studied in living muscle tissue. However, these pathways are less predictable when homeostatic ranges are violated. In addition, there is far less known about how various management or environmental stimuli impact these pathways, either by substrate load or altered cellular environment. Antemortem handling, on the other hand, clearly impacts meat quality development, yet the exact mechanisms remain a mystery. In this paper, we will attempt to review those factors known to affect postmortem energy metabolism in muscle and explore those areas where additional work may be fruitful.

Introduction

Postmortem metabolism is a heavily researched topic. Many scientific and technological advances have led to improved animal welfare, feeding strategies, slaughter processes, and resulted in improved meat quality development for the consumer. Despite a myriad of advances, questions remain regarding those mechanisms controlling or impacting postmortem metabolism and how physiological and tissue-based homeostatic set points are maintained or breeched by various management practices that ultimately lead to altered meat quality development. Though the rate of postmortem metabolism is quite important in driving meat quality development, it is fairly well established. Alternatively, the biochemical mechanism(s) responsible for the cessation of postmortem metabolism, or protracted carbohydrate metabolism are particularly puzzling. Finally, we will briefly review antemortem animal handling practices in an effort to understand how these management practices alter the aforementioned.

Cessation of postmortem metabolism

In order to understand those mechanisms that may control an abbreviated or protracted postmortem metabolism in muscle, one must first reason why it stops. To date, this has not been unequivocally established. Though some would argue it is simply a function of glycogen abundance at harvest, this is not the case, especially when extreme deviates are removed from the population (Copenhafer, Richert, Schinckel, Grant, & Gerrard, 2006; Scheffler & Gerrard, 2007; Scheffler, Park, & Gerrard, 2011). Over sixty years have elapsed since meat scientists across the globe have known that some muscle, for whatever reason, is capable of breaching a final pH, which is otherwise relatively constant across

myriad of animals managed and processed under a variety of conditions, yet little progress or even interest exists in this area. Essentially, there are two viable hypotheses, either there is a pH-mediated inactivation of glycolytic enzymes, which stops hydrogen accumulation at a constant endpoint, or there is loss of adenosine nucleotides preventing a glycolytic substrate to rephosphorylate (Dalrymple & Hamm, 1975; Greaser, 2001). In an attempt to stimulate or re-kindle an interest in this fascinating biochemical process, we will begin by reviewing the collective works of one of the great pioneers of postmortem metabolism, Robert K. Scopes.

Scopes and Lawrie (1963) first entered the area of postmortem metabolism by noting that an accelerated rate of postmortem metabolism resulted in denatured sarcoplasmic proteins and adulterated meat quality development. They predicted at that time that the antemortem 'state' of the animal likely dictated the pH decline in these muscle tissues (Scopes & Lawrie, 1963) and quickly extended these initial observations to show that differences in muscle temperature and pH combinations indeed altered the extractability, or solubility of sarcoplasmic, and myofibrillar muscle proteins (Scopes, 1964). These early data likely formed the foundation for a number of subsequent studies over the next 50 years based on the premise that enzymes, and other proteins, denature with time postmortem (Joo, Kauffman, Kim, & Park, 1999; Warner, Kauffman, & Greaser, 1997), and represent events now commonly known as the conversion of muscle to meat. Most likely because of animal to animal variation, Scopes then modified his approach and used minced muscle to study postmortem metabolism. Using this approach, Newbold and Scopes (1971) showed that varying the concentrations of inorganic phosphate (P_i) up to 50 mM reduced the ultimate pH (pH_u) of the mince, yet concentrations greater than 50 mM

did not further facilitate greater declines in the metabolizing tissue preparation. Based on these observations, they proposed that P_i may induce greater glycogen phosphorylase (GP) activity, thereby explaining the lower pH_u observed in some muscles. This hypothesis is particularly intriguing given the intimate relationship between the phosphagen system and energy metabolism in exercising muscle (Robergs, Ghiasvand, & Parker, 2004). Recall, GP is present in muscle in two forms, *a* and *b*, the former being more active than the latter (Berg, Tymoczko, & Stryer, 2007). GP is activated by AMP and inhibited by ATP and glucose 6-phosphate. Both calcium and epinephrine are capable of shifting the inactive form *b* to the active *a* form by stimulating phosphorylase kinase. Of particular significance is that though active, GP cannot generate glucose 1-phosphate without inorganic phosphate (Morgan & Parmeggianiani, 1964). Given that added inorganic phosphate to a muscle mince caused a lower pH_u in glycolysing muscle preparations (Newbold and Scopes, 1971), it is possible to argue that inorganic phosphate may be rate-limiting during particular times postmortem metabolism. Addition of inorganic phosphate, on the other hand, would undoubtedly raise the pH of the mince early and ultimately could result in a higher pH of the reaction. After all, liberation of free phosphate via ATP hydrolysis, especially in heavily exercising muscle, is known to buffer muscle cells against massive hydrogen accumulation (Robergs, 2001). Regardless, defining the role of phosphate, especially free phosphate in postmortem muscle, may be quite enlightening.

Scopes further refined his in vitro system to include a glycolysing mixture (glycogen, ATP, NAD, P_i, etc.), organic buffers (TRIS, acetate), and purified glycolytic enzymes (Scopes, 1973). The composition of the buffer is particularly germane to the issue at hand, as buffer capacity of muscle can dramatically impact the pH_u and quality of meat

(Kylä-Puhju, Ruusunen, Kivikari, & Puolanne, 2004; van Laack, Kauffman, & Greaser, 2001). Using this system, Scopes (1973) documented the extent resting muscle could rephosphorylate creatine dependent upon available inorganic phosphate, GP *a* concentration, and ATPase activity. He then showed that the rate of glycolysis is directly proportionally to the amount of ATP consumed (Scopes, 1974a). Specifically, when ATPase activity was stimulated, lactate formation was increased proportionally. In addition, he noted that glycolysis stopped once adenonucleotides were metabolized. In contrast, when ATPase, or ATP consumption, was reduced or minimized, the entire system was capable of maintaining ATP concentrations in a steady-state condition, where minimal AMP is detected. As a result, a slower metabolism ensued. These results are particularly interesting as they argue that energy levels such as: phosphocreatine, ATP or the ability of ATP to be rephosphorylated (see discussion below) in the muscle tissue at harvest may shift the time at which glycolysis may begin, or even reaches maximal levels. Changes in the time at which these events occur postmortem could have dramatic effects on ultimate meat quality development, as protein denaturation again is a pH-temperature phenomenon (Offer, 1991; Wismer-Pedersen, 1959). Furthermore, these data show theoretically, that removal of adenonucleotides from glycolysing muscle will arrest metabolism raising another point of control that will be discussed briefly below.

His final and arguably the most important data using this in vitro system, were those directly targeted at understanding the enzymes responsible for pacing both early postmortem metabolism and that responsible for extending carbohydrate metabolism in skeletal muscle (Scopes, 1974b). These data showed that regardless of enzymes present, ATPase concentration, or ATP consumption, drives the rate of metabolism. These findings

formed the basis by which many understand the role ATPase plays in controlling the rate of postmortem metabolism (Bowker, Grant, Swartz, & Gerrard, 2004; Hamm, 1977), especially where inherent differences in ATP consumption found between muscles of different fiber types change postmortem metabolism (Fernandez & Tornberg, 1991; Klont, Brocks, & Eikelenboom, 1998). Moreover, early postmortem consumption of ATP in muscle is hallmark of halothane-positive pigs containing a mutated calcium channel protein that allows cellular calcium concentrations to rise to a point where corresponding downstream ATPases force an aggressive metabolism and abberant meat quality development (Cheah, Cheah, Crosland, Casey, & Webb, 1984; Greaser, Cassens, Briskey, & Hoekstra, 1969; Monin, Sellier, Ollivier, Goutepongea, & Girard, 1981). Results of these studies also raised the idea that GP *a*, and to a lesser extent AMP deaminase concentrations may dictate the pH at which metabolism stops (Scopes, 1974b).

Understanding how Scopes settled on GP *a* as a driver of pH_u in porcine skeletal muscle is logical though difficult to explain in living, or dying muscle. In his studies (Scopes, 1974b), GP *a* concentrations were included at sub- and supra-physiological levels. While the increased levels of GP *a* resulted in a reduced pH_u in vitro, it also accelerated the rate of pH decline, begging the question of whether phosphorylase differs between pigs with altered muscle metabolism. GP *a* activity in resting halothane positive pig muscle is lower compared to wild-type pigs (Fernandez, Neyraud, Astruc, & Sante, 2002), yet increases above that of wild-type animals during postmortem metabolism (Monin, Talmant, Laborde, Zabari, & Sellier, 1986). This increase in phosphorylase *a* content is likely due to the calcium-mediated activation of phosphorylase kinase, the enzyme responsible for the conversion of GP *b* to *a* form (Meyer, Fischer, & Krebs, 1964).

Alternatively, it could be due to increased catecholamine release and action at harvest (Althen, Ono, & Topel, 1977). However, classically halothane pigs do not differ in pH_u (Copenhafer, et al., 2006; De Smet, et al., 1996; Fernandez, et al., 2002; Klont, Lambooy, & van Logtestijn, 1993; Kocwin-Podsiadla, Przybylski, Kuryl, Talmant, & Monin, 1995) which argues against GP *a* content driving the pH_u of meat. Even so, a number of investigators have shown that meat of halothane positive pigs results in a lower pH_u (Fisher, Mellett, & Hoffman, 2000; Hamilton, Ellis, Miller, McKeith, & Parrett, 2000; Klont & Lambooy, 1995; Klont, Lambooy, & van Logtestijn, 1994; Monin, et al., 1981). The latter issue makes it difficult to study cessation of postmortem metabolism in halothane-sensitive pigs. In comparison, no differences have been noted in GP *a* activity between wild-type and Rendement Napole (RN) pigs (Estrade, Ayoub, Talmant, & Monin, 1994). Recall, RN pigs possess a gene mutation that somehow allows for a breech in the normal postmortem set points and results in lower pH_u and a type of 'acid meat'. Regardless, the aforementioned data strongly support the notion that GP may be involved in controlling the extent of postmortem metabolism and should be closer scrutinized.

Though not specifically addressed by Scopes' work, the enzyme most frequently implicated as responsible for the cessation of postmortem metabolism is phosphofructokinase (PFK) (Bendall, 1973; Hamm, 1977), due in part, to its rate-limiting status in glycolysis and its complex control in living tissues (Berg, et al., 2007). Moreover, glucose 6-phosphate increases late postmortem (Copenhafer, et al., 2006; Kastenschmidt, Hoekstra, & Briskey, 1968) suggesting a loss in PFK activity sometime earlier. Data to directly support this hypothesis are scant. However, a recent study comparing normal and RN pig muscle showed a number of sarcoplasmic and myofibrillar proteins experience

phosphorylation events postmortem (Lametsch, et al., 2011). To that end, the increased phosphorylation of PFK in RN pig muscle may increase its stability and subsequent pH inhibition during the postmortem period (Sola-Penna, Da Silva, Coelho, Marinho-Carvalho, & Zancan, 2010). This may help explain the lower pH_u of fresh pork derived from this genotype.

Schwägele & Honikel (1988) quantified a host of glycolytic enzyme activities over a wide range of pH values (5.3 to 6.8) found in postmortem tissue. Results showed the activities of PFK, glyceraldehyde 3–phosphate dehydrogenase, phosphoglycerate kinase, pyruvate kinase, and adenylate kinase varied with pH conditions studied; however no enzyme lost activity completely. Enzymes were extracted in a phosphate buffer (pH 7.0) suggesting assays were conducted at a pH close to the physiological norm, rather than those conditions found postmortem. If this was indeed the case, then the activity of these enzymes at normal postmortem conditions remains unknown, though Scopes argues that PFK remains active at pH 5.35 (Scopes, 1974b). Regardless, these findings provide some evidence that glycolytic-based enzyme inactivity may be subtle. These data do not, however, rule-out enzyme inactivation as a means of arresting postmortem metabolism.

The idea that postmortem metabolism stops in response to adenonucleotides loss is intriguing and rational. After all, the enzymes thought to be rate-limiting in glycogenolysis and glycolysis (PFK, GP, pyruvate kinase) are all allosterically activated/inactivated by ATP or its metabolites (Berg, et al., 2007). Under normal circumstances, ATP is hydrolyzed to ADP, which then can be rephosphorylated to ATP by either phosphoglycerate kinase, pyruvate kinase or adenylate kinase. Adenylate kinase converts 2 ADP molecules into one ATP and one AMP molecule ($ADP + ADP \rightarrow ATP + AMP$)

(Pearson, 1971). Once formed, AMP is quickly converted by AMP deaminase (AMP → IMP) and unavailable for use by glycolysis (Pearson & Young, 1989), thus reducing the concentration of the adenonucleotide pool and eliminating a substrate for glycolysis to phosphorylate. The adenylate kinase and AMP deaminase reactions are coupled so an increase in activity of one results in an increase of the other. Though subtle differences exist between species (Fishbein, Davis, & Foellmer, 1993), AMP deaminase or adenylate kinase activity do not differ greatly, mitigating excitement for either in controlling differences in the extent of postmortem metabolism postmortem. Even so, these enzymes likely deserve some additional consideration regarding our quest to understand fully postmortem metabolism.

One means of testing whether adenonucleotides dictate the cessation of glycolysis would be to eliminate them experimentally from the tissue. A complete loss of ATP would necessarily stimulate adenylate kinase and AMP deaminase and speed up the arrest of glycolysis. The only approach readily available is electrical stimulation, which stimulates glycolysis (Hwang, Devine, & Hopkins, 2003). Theoretically, it may be possible to hydrolyze enough ATP that sufficient energy would not be available to break rigor bonds, and thereby arrest glycolysis. Then, is it possible to stimulate carcasses with electricity enough to stop postmortem metabolism? If so, would sustained electrical stimulation create a dark, firm, and dry (DFD)-like meat condition if the loss of adenosine nucleotides is solely responsible for the cessation of glycolysis and establishment of the pH_u of meat? Across numerous electrical stimulation studies reviewed, we have yet to read of any form of electrical stimulation that ever resulted in higher pH_u values or DFD-like meat, at least in pigs (Hallund & Bendall, 1965; Hammelman, et al., 2003; Maribo, Ertbjerg, Andersson,

Barton-Gade, & Møller, 1999; Taylor, Nute, & Warkup, 1995). In fact, electrical stimulation is a reliable tool to produce pale, soft, and exudative (PSE) meat in pigs (Bowker, Grant, Forrest, & Gerrard, 2000). Taken together, these data indicate that despite a rapid loss of ATP caused by electric stimulation, sometimes repeated, enough nucleotides remain in the muscle to facilitate glycolysis to a normal pH_u. Even so, until we find a way to eliminate ATP experimentally, we cannot rule out this hypothesis.

Antemortem handling

Feed withdrawal or fasting prior to slaughter is commonly used to improve meat quality by elevating pH_u through a reduction in liver (Warriss, 1982) and muscle glycogen (Fernandez & Tornberg, 1991; Sterten, Oksbjerg, Frøystein, Ekker, & Kjos, 2010; Wittmann, Ecolan, Levasseur, & Fernandez, 1994). A Bayesian meta-analysis using 16 studies showed fasting duration increases the pH_u of the *longissimus* muscle of pigs (Salmi, et al., 2012) yet, the correlation is weak likely due to the unique relationship between glycogen and the pH_u of meat. In fact, this relationship is curvilinear where the muscle glycogen content is primarily responsible for dictating pH_u between a pH_u of 7.2-5.7 (Bendall, 1973; Henckel, Karlsson, Jensen, Oksbjerg, & Petersen, 2002), but below 5.7, other as yet unknown mechanisms must be responsible for the variation in this range. Some of the possible mechanisms have already been discussed in earlier sections, yet others likely remain.

During normal postmortem metabolism, the *longissimus* muscle (white, glycolytic muscle) utilizes 35-40 μmol glycogen/g tissue to achieve a pH_u near 5.5 (Copenhafer, et al., 2006). Oftentimes, this muscle contains greater than necessary amounts of glycogen

(60 μmol/g) resulting in 2-30% of the total glycogen remaining at 24 h (Pearson & Young, 1989). This residual glycogen content may help explain the weak relationship between fasting and pH_u. Fasting reduces glycogen content of muscle by as much as 20% in the *longissimus* muscle of pigs over a 24 h period (Wittmann, et al., 1994), yet this reduction is insufficient to reduce glycogen below the aforementioned threshold that affects pH_u. Henckel et al. (2002) suggests that increases in pH_u will not develop unless muscle glycogen is reduced below 53 μmol/g tissue supporting this argument. Furthermore, red, oxidative muscles contain less glycogen and produce meat with higher pH_u (Monin & Talmant, 1987). Curiously, a 24 h fast reduces the glycogen content of the more oxidative *semispinalis* muscle by 50% and results in a higher pH_u (Wittmann, et al., 1994). Thus, the percentage decrease in glycogen between the two muscles in response to a 24 h fast is quite different, but the absolute decrease of each is quite similar (~ 15 μmol/g) (Wittmann, et al., 1994). Therefore, fasting may reduce the stored carbohydrate content of muscle below this 'critical' threshold and thus, fasting may be more effective on red muscles due to their lower resting glycogen content. This is especially apparent with RN pigs. Again, RN pig muscle contains extremely high levels of glycogen and produce meat with low pH_u. When fasted for 36 h, the pH_u of meat from RN mutant pigs still remained lower than controls (Bidner, Ellis, Witte, Carr, & McKeith, 2004), arguing that a 36 h fast is not sufficient to lower glycogen levels below the critical threshold where it can impact pH_u and recover a normal phenotype. Regardless of whether fasting impacts postmortem metabolism and meat quality characteristics, the practice of fasting should not be eliminated from the standard slaughter procedure because of the microbiological

ramifications mitigated by reducing gastrointestinal tract content (Miller, Carr, Bawcom, Ramsey, & Thompson, 1997).

Of particular significance to the issue at hand is the idea that different sources of dietary energy impact antemortem muscle glycogen concentrations (reviewed by Andersen, Oksbjerg, Young, & Therkildsen, 2005). Most intriguing is the idea that easily digestible carbohydrates may alter postmortem metabolism to prevent DFD meat. Muscle of pigs fed a diet of sucrose resulted in an accelerated pH decline, lower pH_u (Briskey, Bray, Hoekstra, Phillips, & Grummer, 1959) and elevated glycogen (Briskey, Bray, Hoekstra, Phillips, & Grummer, 1960). To explain the accelerated metabolism, a follow-up study compared the GP activity between control fed and sucrose pigs, but found no difference between the two treatments were detected (Sayre, Briskey, & Hoekstra, 1963). High-sugar diets encourage the incorporation of carbohydrate into the muscle as glycogen and alters glucose metabolism (reviewed by Fernandez and Tornberg, 1991) which may alter the enzymatic activity within the muscle. Though GP activity was not altered in sucrose-fed pigs (Sayre, et al., 1963), other enzymes may have been altered to facilitate an accelerated metabolism. Alternatively, a lower pH_u in meat may have been directly a result of increased glycogen deposition. However, very high inclusion rates may be necessary to achieve this endpoint and this alone may have detrimental effects on tissue energy metabolism as up to 15% sucrose incorporation to grower and finishing pig diets has no effect on glycogen content or postmortem metabolism (Camp, Southern, & Bidner, 2003).

Regardless of the mechanism, diet can be used to mitigate low pH values by reducing carbohydrates from the diet to less than 5% and increasing fat to approximately 18% on a weight basis (Rosenvold, et al., 2001). When fed three weeks prior to slaughter,

these diets reduce the glycogen content of the *longissimus* dorsi by 11-26% (Rosenvold, et al., 2001) and increase pH_{45} and pH_u (Rosenvold, et al., 2002; Tikk, Tikk, Karlsson, & Andersen, 2006) and accelerate temperature decline (Tikk, Lindahl, Karlsson, & Andersen, 2008; Tikk, et al., 2006). Presumably, this feeding strategy does not alter key glycolytic enzymes like PFK, GP *a*, or GP *b* activity, but may lower μ-calpain activity (Rosenvold, et al., 2002), the enzyme responsible for postmortem proteolysis of myofibrillar proteins (Huff-Lonergan & Lonergan, 2007).

Strategic feeding protocols and fasting are intriguing because both appear to reduce glycogen content, which are thought to improve meat quality, yet there is no indication of an interaction between the two. To our know, only one study has been conducted to address this issue (Partanen, Honkavaara, & Ruusunen, 2007). In this study, the longer fasting time and a fibrous diet increased pH_u and lowered muscle glycogen, and indicated that the combination of the two may produce an additive effect to elevate pH_u.

Pre-slaughter stress is complicated, but clearly reduces meat quality through altered metabolism. If an animal is stressed long-term, glycogen stores may be depleted resulting in abbreviated metabolism and a high pH_u (Briskey, 1964). Adrenaline injections prior to slaughter can produce a similar result if administered appropriately (Bendall & Lawrie, 1962; Henckel, et al., 2002; Henckel, Karlsson, Oksbjerg, & Petersen, 2000; Rosenvold, et al., 2001). Long-term stress requires a recovery period known as lairage. From a tissue standpoint, this is interpreted as sufficient time to re-establish the glycogen that was in the muscle, or eliminate entirely the stress hormones, mostly catecholamines, that may have been released in the body in response to the stress (Faucitano, 2010). For the most part, increased lairage times elevate pH_{45}, pH_u and thereby improve meat quality measurements,

especially L* (lightness) values (Salmi, et al., 2012; Zhen, et al., 2013). Alternatively, short-term stress immediately prior to slaughter is known to accelerate metabolism and result in higher muscle temperatures and a lower pH_u (van der Wal, Engel, & Reimert, 1999), a condition similar to the HAL mutation. Short-term stress is difficult to remedy due to its occurrence immediately prior to slaughter during handling or movement of the animal to the abattoir. Rosenvold and Andersen (2003) provide a much more comprehensive review about the differences between long-term and short-term stress upon postmortem metabolism.

To complicate matters further, the effects can be combined. Shen et al. (2006) induced pre-slaughter transport stress upon pigs both with and without rest. Transport resulted in decreased (glycogen + glucose + G6P) early postmortem as well as reduced muscle pH. Muscle from transported pigs, without rest, produced meat with a lower pH_u compared to control animals, exhibited an accelerated (AMP + IMP) / ATP ratio, maintained higher GP *a* activity through 4 h postmortem, and resulted in meat with a higher drip loss and a PSE condition. While muscle glycogen was indeed reduced in these animals, it was insufficient to produce DFD meat and only resulted in accelerated metabolism. Recall, muscle glycogen must be reduced below 53 μmol/g tissue to produce DFD meat. A general lack of consensus reflects the complexity of studying the effect of transportation on ultimate energy metabolism in the muscle, especially in pigs. Even so, it argues that pre-slaughter stress is capable of producing both DFD and PSE meat, yet the exact biochemical mechanisms remain quite unclear.

Conclusions

When thinking about the conversion of muscle to meat, unanswered questions still remain. The mechanism responsible for the cessation of anaerobic glycolysis postmortem has eluded researchers for the past 60 years, but a pH-mediated inactivation of glycolytic enzymes or a loss of adenosine nucleotides is likely. Finally, prior to slaughter, the energy charge created in the animal's muscles dictates the rate and extent of metabolism. Fasting, diet, transport and lairage all affect postmortem metabolism and must not be overlooked when attempting to improve meat quality.

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Chapter 2 - pH inactivation of phosphofructokinase arrests postmortem glycolysis

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Abstract

Fresh meat quality development is influenced by pH decline that results from muscle glycolyzing energy substrates postmortem. The exact reason for why glycolysis stops in the presence of residual glycogen remains unclear. We hypothesized that a critical glycolytic enzyme loses activity near the ultimate pH of meat. Porcine *longissimus muscle* samples were subjected to an *in vitro* system that mimics postmortem anaerobic metabolism at buffered pH values (7.0, 6.5, 6.0, 5.5 or 5.0). At pH 7.0, 6.5, and 6.0, glycogenolysis and glycolysis proceeded normally while pH 5.5 stopped lactate formation. Additional experimentation indicated that phosphofructokinase lost activity at pH 5.5 while all other glycolytic enzymes remained active. A similar inactivation of phosphofructokinase was observed when using chicken and beef muscle. Elevated temperature hastened pH decline and phosphofructokinase activity loss. Thus, pH

inactivates phosphofructokinase and arrests postmortem glycolysis, which may explain the similar ultimate pH across meat of different species.

Introduction

During the conversion of muscle to meat, a myriad of biochemical pathways are invoked in a futile attempt to retain energy at resting, homeostatic, pre-harvest set-points. Every time a molecule of ATP is hydrolyzed, a hydrogen ion (H^+), ADP and an inorganic phosphate (P_i) molecule are liberated ($ATP \rightarrow ADP + H^+ + P_i$) by ATPase. Thus, the rate of postmortem pH decline is controlled by ATPase activity (Bowker, Botrel, Swartz, Grant, & Gerrard, 2004; Bowker, Grant, Swartz, & Gerrard, 2004; Hamm, 1977; Newbold & Scopes, 1971a, 1971b; Scopes, 1974a, 1974b). Yet, the mechanisms responsible for extending metabolism and reducing the ultimate pH of meat remain unresolved.

As postmortem metabolism proceeds under anaerobic conditions, flux through glycolysis remains the sole means of rephosphorylating ADP. Substrates, in the form of glucose 6-phosphate (G6P), must be provided to support this anaerobic-enabling pathway. Though small amounts of G6P may be supplied initially through the actions of hexokinase and ATP, the bulk of G6P originates from the liberation of glycogen to glucose 1-phosphate and its subsequent conversion to G6P. This latter ATP-independent process only occurs in the presence of sufficient P_i, which conveniently increases with greater ATP consumption (Robergs, Ghiasvand, & Parker, 2004).

As elegant as the energy system is, it fails postmortem raising the question, why does glycolysis stop? On the surface, the most logical reason is that postmortem glycolysis occurs until glycogen stores are depleted. Yet, metabolizable carbohydrate is rarely

depleted entirely in muscle (Bendall, 1973; Copenhafer, Richert, Schinckel, Grant, & Gerrard, 2006; Henckel, Karlsson, Jensen, Oksbjerg, & Petersen, 2002; Immonen & Puolanne, 2000). Monin and Sellier (1985) first observed that some pigs accumulated abnormally high levels of muscle glycogen and produced meat with an abnormally low ultimate pH known as acid pork (Milan, et al., 2000). This led to the hypothesis that elevated glycogen results in prolonged glycolysis postmortem and subsequently resulted in a lower ultimate pH in some pork. This construct was supported by showing a strong relationship between initial muscle glycogen content and the ultimate pH of meat (Josell, et al., 2003; Warriss, Bevis, & Ekins, 1989; Yla-Ajos, et al., 2007). This whole notion was preempted by the classic work of Briskey (1964) where he plotted muscle pH against time postmortem. He used a heterogeneous population of pigs and showed that at one end of the spectrum dark, firm and dry (DFD) pork had abnormally high ultimate pH values, while acid pork had low values. Because animals with depleted glycogen experience little glycolysis, a cause and effect relationship between muscle glycogen content antemortem and ultimate muscle pH was particularly attractive, at least for DFD meat (Bendall, 1973; Briskey, 1959). Given this fact alone, it was logical to assume that a lower than normal ultimate pH in muscle arises from extended glycolysis facilitated by excessive glycogen storage. Yet, unequivocal data supporting such a relationship are lacking.

Therefore, given that ultimate pH of muscle is quite consistent, even across species, argues a strong biochemical foundation for arresting glycolysis in muscle postmortem. Termination of glycolysis is thought to occur when muscle tissue reaches a particular pH and inactivation of some critical glycolytic enzyme occurs, or a substrate(s) such as ADP, NAD+ and/or P_i becomes limiting (Bendall, 1973; Dalrymple & Hamm, 1975; Greaser,

2001; Hamm, 1977; Kastenschmidt, Hoekstra, & Briskey, 1968). Though, it is more likely a combination of the aforementioned mechanisms, while glycolytic flux may be substrate- and energy-dependent, it is only so until a particular pH is reached. This led us to postulate that a critical glycolytic enzyme must lose function at a pH just above the normal ultimate pH of meat.

Phosphofructokinase (EC 2.7.1.11; PFK) is a particularly attractive enzyme for arresting glycolysis due to its role as the committed step of glycolysis. PFK is a 340 kDa tetrameric protein comprised of muscle, liver or platelet specific subunits based on tissue localization (Dunaway, 1983). Maximal glycolytic flux requires full activation (Berg, Tymoczko, & Stryer, 2007) and the enzyme undergoes an oligomerization process where it shifts from a tetramer to its less active dimeric or monomeric forms (Sola-Penna, Da Silva, Coelho, Marinho-Carvalho, & Zancan, 2010). Lactate initiates dissociation of native PFK into its less active dimeric form and the effect is enhanced by reducing pH (Costa Leite, Da silva, Guimarães Coelho, Zancan, & Sola-Penna, 2007). Moreover, accumulation of G6P was noted by a number of investigators late postmortem in pig muscle (Copenhafer, et al., 2006; Hammelman, et al., 2003; Kastenschmidt, et al., 1968) and beef muscle (Fabiansson & Laser Reuterswärd, 1986; Nuss & Wolfe, 1981).

PFK is also tightly controlled by up- and down-stream substrates and metabolites. Inhibitors of PFK include ATP, lactate and citrate while stimulators include ADP, AMP, calcium and fructose 2,6-bisphosphate (F-2,6-BP). F-2,6-BP and the enzyme responsible for its production, phosphofructokinase-2 (EC 2.7.1.105; PFK2), were studied extensively due to their interaction with AMP-activated protein kinase (AMPK) (Shen & Du, 2005; Shen, Gerrard, & Du, 2008; Shen, et al., 2006; Shen, Underwood, Means, McCormick, &

Du, 2007). Yet, PFK2 is distinct from PFK and is primarily responsible for regulating glycolysis and gluconeogenesis in the liver (Okar, et al., 2001). While the exact role of PFK2 postmortem is unknown, we chose to focus on PFK mainly because of its predominance and function in skeletal muscle. To that end, we used frozen, powdered muscle excised early postmortem (within minutes of exsanguination) and suspended these samples in an *in vitro* muscle glycolytic system (Scopes, 1973) to simulate postmortem glycolysis. Using this approach, we were able to use the tissue as the source of glycolytic enzymes and determine how these enzymes functioned as glycolysis proceeded under different environmental constraints.

Materials and methods

Animals and Sample Preparation

Market-weight pigs, cattle, and chickens were harvested in the Virginia Tech Meat Center using accepted commercial processing procedures. Following exsanguination, porcine *longissimus thoracis et lumborum muscle* (LTL) samples were excised at 5 min postmortem, snap frozen in liquid nitrogen, and stored at -80 °C. Chicken *pectoralis major* (glycolytic tissue), beef *semimembranosus*, and pork *semitendinosus* (oxidative tissue) were excised and stored in a similar manner at 5, 15 and 20 min postmortem, respectively. Porcine LTL was used in all experiments while the chicken, beef and porcine *semitendinosus* were only used to determine PFK activity.

In Vitro Buffer System

Porcine LTL samples were powdered in liquid nitrogen and homogenized at a 1:10 ratio (wt/ vol) with a modified reaction buffer adjusted to static pH values 7.0, 6.5, 6.0, 5.5 and 5.0 (7 pigs) or pH values 6.0, 5.9, 5.8, 5.7, 5.6 and 5.5 (3 pigs). The buffer contained 200 mM 2-(N-Morpholino) ethanesulfonic acid (MES), 10 mM Na_2HPO_4, 5 mM $MgCl_2$, 60 mM KCl, 5 mM ATP, 0.5 mM ADP, 0.1 mM AMP, 0.5 mM NAD+, 15 mM glycogen, 25 mM carnosine, 30 mM creatine, 10 mM glucose 6-phosphate, 1 mM fructose 1,6-bisphosphate, and 10 mM sodium acetate. Reaction vessels were stored at 25 °C with shaking, and aliquots were removed at 0, 120, 240, 360 and 1440 min for analysis.

In a separate experiment, porcine LTL from 6 animals was powdered in liquid nitrogen and homogenized at a 1:10 ratio (wt/ vol) in a reaction buffer containing 10 mM Na_2HPO_4, 5 mM $MgCl_2$, 60 mM KCl, 5 mM ATP, 0.5 mM ADP, 0.5 mM NAD+, 15 mM glycogen, 25 mM carnosine, 30 mM creatine, 10 mM glucose 6-phosphate and 10 mM sodium acetate. Reaction vessels were stored in a dry block heater at 33, 36, 39 and 42 °C for the duration of the trial. Aliquots of the muscle homogenate were taken at 0, 30, 60 120, 180 and 240 min and added directly to the PFK activity assay buffer or pH solution and quantified.

pH Analysis

Measurement of pH was conducted according to Bendall (1973) with slight modification. Four volumes of homogenate were removed from the buffer system and one volume of 25 mM sodium iodoacetate and 750 mM KCl (pH 7.0) was added. After centrifugation, samples were equilibrated at 25 °C and measured using an Orion Ross Ultra pH electrode (Thermo Scientific, Pittsburgh, PA). The probe was calibrated each day prior to analysis.

Metabolite Analysis

Aliquots for glycogen analysis were removed from the homogenate, mixed with an equal volume of 2.5 M HCl, heated at 90 °C for 2 hr, centrifuged at 13,000 x g for 5 min and neutralized with 1.25 M KOH (Bergmeyer, 1984). Separate muscle homogenate samples were removed for lactate, glucose 6-phosphate and adenonucleotide analysis. The aliquot was treated with an equal volume of ice-cold 1M perchloric acid, centrifuged at 13,000 x g for 5 min and the supernatant was neutralized with 2 M KOH. (Bergmeyer, 1984). Lactate, glucose 6-phosphate and glycogen concentrations were measured (Bergmeyer, 1984) using a method modified for microplate volumes (Hammelman, et al., 2003). All reactions occurred in borosilicate glass tubes and were analyzed spectrophotometrically at 340 nm in triplicate using 96-well microplates. Adenonucleotides were separated with an Accucore C18 2.6 u 50 x 4.6 mm column (Thermo Scientific, Pittsburgh, PA) and detected at 254nm with an HP Agilent 1100 (Agilent Technologies, Santa Clara, CA) using gradient separation (Bernocchi, et al., 1994; Williams, Vidt, & Rinehart, 2008).

Phosphoglucoisomerase Activity Assay

LTL samples from 3 pigs were homogenized in 100 mM K_2HPO_4 (pH 7.4) at a 1:3 (wt/ vol). Aliquots of the muscle homogenate were added to a reaction buffer containing 200 mM MES, 10 mM Na_2HPO_4, 5 mM $MgCl_2$, 60 mM KCl, 0.5 mM NAD+, 25 mM glycogen, 25 mM carnosine, 30 mM creatine, 10 mM glucose 6-phosphate and 10 mM sodium acetate with a static pH (5.0, 5.5 or 6.0). Aliquots were taken at 0, 10, 30 and 60 min and stopped with an equal addition of ice-cold 1M perchloric acid. Samples were centrifuged at 13,000

x g for 5 min and the supernatant was neutralized with 2M KOH. Fructose 6-phosphate was analyzed according to Bergmeyer (1984) to determine enzyme functionality.

Fructose-Bisphosphate Aldolase To Lactate Dehydrogenase Activity Assay

LTL samples from 3 pigs were homogenized in 100 mM K_2HPO_4 (pH 7.4) at a 1:3 ratio (wt/ vol). Aliquots of the muscle homogenate were added to a reaction buffer containing 200 mM MES (pH 5.0 or 5.5), 60 mM KCl, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 2 mM ATP, 0.5 mM ADP, 0.1 mM AMP, 0.5 mM NAD^+, 25 mM carnosine, 30 mM creatine and 10 mM sodium acetate. The treatment group contained 4 mM fructose 1,6-bisphosphate (F-1,6-BP) while the control treatment did not. Aliquots were taken at 0, 180, 360 and 1440 min for lactate quantification as previously described.

Glycogen Phosphorylase Activity Assay

Glycogen phosphorylase *a* and total (*a+b*) activity were analyzed from 5 pigs according to Bergmeyer (1971). Briefly, muscle tissue was homogenized into ice-cold 0.1M K_2HPO_4 (pH 7.4) and an aliquot of the homogenate was added to the reaction buffer containing 50 mM K_2HPO_4, 2 mg/mL glycogen, 1.3 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM NADP, 200 mM MES (pH 6.8), 1 U/mL phosphoglucomutase and 1 U/mL glucose 6-phosphate dehydrogenase to measure glycogen phosphorylase a activity. Glycogen phosphorylase *a+b* activity was also quantified in the same buffer with the addition 1.75 mM AMP. Activity was measured in triplicate using 96-well microplates spectrophotometrically at 340 nm and reported as μmol NADPH * min^{-1} * g^{-1}.

Phosphofructokinase Activity Assay

Phosphofructokinase activity from 5 pigs was determined according to Trivedi & Danforth (1966). Muscle samples were homogenized in ice-cold 0.1M K_2HPO_4 (pH 7.4) and aliquots of the homogenate were added to the reaction buffer containing 120 mM MES (pH 6.5), 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. Enzymatic activity was measured in triplicate spectrophotometrically at 340 nm and reported as μmol NADH * min^{-1} * g^{-1}. PFK activity was similarly determined at pH 5.6 or 6.5 with or without 0.15 mM AMP. Activity was compared between 3 animals each of pork LTL, chicken *pectoralis major*, beef *semimembranosus*, and pork *semitendinosus*.

Pyruvate Kinase Activity Assay

Pyruvate kinase activity from 5 pigs was measured according to Feliu, Hue, & Hers (1976). Muscle tissue was homogenized into ice-cold 0.1M K_2HPO_4 (pH 7.4) and added to a buffer containing 120 mM MES (pH 6.5), 100 mM KCl, 10 mM $MgCl_2$, 1.25 mM ADP, 1 mM NADH, 0.5 mM phosphoenolpyruvate (PEP) and 2 U/mL lactate dehydrogenase. Disappearance of PEP was quantified with a spectrophotometer at 340 nm and reported as μmol PEP * min^{-1} * g^{-1}.

Statistical Analysis

Data are presented as means ± SE. Data were analyzed with a mixed model in JMP (SAS Institute Inc., Cary, NC). Each animal was recognized as an experimental unit and time course data were analyzed with a split-plot design. Least-square means were evaluated

using a student's t-test and considered significant at $P \leq 0.05$. Data from the experiment comparing PFK activity between species were compared within the species/muscle combination and data from the temperature experiment were compared within time.

Results and discussion

pH Inactivation of glycolytic and glycogenolytic enzymes

In our initial experiment, we studied the impact of pH (7.0, 6.5, 6.0, 5.5 and 5.0) on *in vitro* postmortem glycolysis. To accomplish this, 200 mM MES**,** a biological buffering agent (pK_a = 6.15), was added to our system which maintained the specific pH values throughout the entire duration of the study (data not shown). At pH 7.0, 6.5 and 6.0, glycogenolysis and glycolysis occurred like normal muscle as evident by the disappearance ($P < 0.0001$) of glycogen and G6P and increased ($P < 0.0001$) lactate with time (Figure 2-1). Enzymatic inactivation was not evident at these pH treatments, though a reduced ($P < 0.05$) rate of glycogen and G6P disappearance and lactate formation was noted at pH 6.0. In contrast to the higher pH treatments, pH 5.5 resulted in increased ($P < 0.0001$) G6P formation with time (Figure 2-1B) and indicates both glycogen phosphorylase and phosphoglucomutase function at this lower limit of muscle pH.

At pH 5.0, lactate accumulation was not detected ($P \geq 0.35$) with time, though a small increase ($P = 0.01$) was noted in pH 5.5, specifically from 1.50 ± 0.28 mM at 0 min to 3.98 ± 0.63 mM at 1440 min (Figure 2-1C). This small increase was likely due to the included 2 mM fructose 1,6-bisphosphate. In addition, the sum total of glycogen, G6P, fructose 6-phosphate and glucose wase determined at 0 and 1440 min (Table 2-1) and did not decrease

($P \geq 0.50$) with time in the pH 5.0 and 5.5 treatment. These results indicated that an enzyme in glycolysis fails to function between pH 5.5 and 6.0. To determine the exact pH where lactate formation ceased, we titrated pH at 0.1 increments between pH 5.5 and 6.0. At pH 5.6 - 6.0, lactate increased ($P \leq 0.04$) at both 3 and 24 hr (Figure 2-2), but no increases were detected at pH 5.5.

These experiments show that glycolysis begins to slow at pH 6.0 and ceases to function at or very near pH 5.5. Further, these results also show that glycogen phosphorylase and phosphoglucomutase function at a pH of 5.5, but one or both may lose activity below this value. Therefore, glycogen phosphorylase and phosphoglucomutase are not responsible for the cessation of glycolysis in our *in vitro* system.

Glycolytic enzyme activity assays

We further confirmed the enzyme(s) responsible for arresting postmortem glycolysis. The enzyme immediately following phosphoglucomutase in glycolysis is phosphoglucoisomerase. At all pH values studied (6.0, 5.5 and 5.0), G6P was converted to fructose 6-phosphate (Figure 2-3). We also noted that the G6P to fructose 6-phosphate equilibrium in these studies was approximately 3:1, which is consistent with values reported by Kahana, Lowry, Schulz, Passonneau, & Crawford (1960) and supports the utility of our *in vitro* system to simulate the *in vivo* environment. These data show that phosphoglucomutase functions at pH values lower than the ultimate pH of pork and suggest it is not responsible for arresting glycolysis in postmortem muscle.

The remaining glycolytic enzymes (excluding PFK) that could have been responsible for our observations are aldolase to lactate dehydrogenase. To test the collective functionality

of these enzymes, commercially acquired F-1,6-BP was added to our system at pH 5.5 and 5.0. In both cases, F-1,6-BP was converted to lactate (Figure 2-4). These data show the enzymes from aldolase to lactate dehydrogenase are capable of functioning to pH 5.0, well below the ultimate pH of pork. In both beef and pork, little residual glycolytic intermediates are detected at rigor between F-1,6-BP and pyruvate (Fabiansson & Laser Reuterswärd, 1986; Kastenschmidt, et al., 1968). Based on these studies, PFK must be responsible for the cessation of postmortem glycolysis because all other glycolytic and glycogenolytic enzymes remain active at or below pH 5.5 in our *in vitro* system.

Postmortem glycolytic and glycogenolytic enzyme activity

To validate of our *in vitro* results *in vivo*, we determined glycolytic enzyme activity from postmortem muscle samples. Porcine muscle tissue was sampled at 0, 30, 60, 120, 240 and 1440 min postmortem to determine the activity changes in glycogen phosphorylase, pyruvate kinase and PFK, the three rate-limiting enzymes of glycolysis. All samples were tested at the same pH for each individual enzyme assay in order to compare activities between postmortem time-points. Activities from pork LTL were similar to previously published reports for glycogen phosphorylase (Monin, Mejenes-Quijano, Talmant, & Sellier, 1987) and pyruvate kinase and PFK (Allison, Bates, Booren, Johnson, & Doumit, 2003; Schwägele, Haschke, Honikel, & Krauss, 1996).

Total glycogen phosphorylase ($a+b$) activity increased slightly from 0 min to 180 ($P = 0.02$) and 240 ($P = 0.02$) min (Figure 2-5A). This is consistent with previous reports that concluded total phosphorylase activity does not change from 60 to 1440 min in porcine muscle (Fischer, Hamm, & Honikel, 1979). Glycogen phosphorylase *a* activity decreased (*P*

< 0.05) with time (Figure 2-5A). Scopes (1974) initially postulated glycogen phosphorylase *a* activity could be responsible for defining the ultimate pH of meat, yet attempts to use glycogen phosphorylase *a* activity as a predictor of ultimate pH have yielded little validation (Estrade, Ayoub, Talmant, & Monin, 1994; Fernandez, Neyraud, Astruc, & Sante, 2002; Schwägele, Buesa, & Honikel, & Krauss, 1996; Talmant, Monin, Briand, Dadet, & Briand, 1986). A more comprehensive analysis of glycogen phosphorylase's role in arresting postmortem glycolysis is included in our review (England, et al., 2013).

Pyruvate kinase activity stayed consistent with time postmortem (Figure 2-5B), though activity spiked ($P = 0.016$) at 180 min. The increase in activity may be caused by additional phosphorylation events of pyruvate kinase (Schwägele, Haschke, et al., 1996), but Allison, et al. (2003) reported pyruvate kinase could not explain variation in pork quality.

We concede that it is possible that inactivation of pyruvate kinase and/or glycogen phosphorylase may contribute to the termination of postmortem glycolysis as either could regain functionality when placed in a neutral buffer. However, the results from the previous two experiments argue against this idea because both enzymes remained active *in vitro* at pH 5.5. Therefore, it is unlikely that glycogen phosphorylase or pyruvate kinase is responsible for halting postmortem glycolysis.

PFK activity, on the other hand, changed dramatically with time postmortem (Figure 2-5C). Initial activity was greater ($P \leq 0.0001$) than 30, 60, 120, 180, 240 and 1440 min postmortem. Werner et al. (2010) showed PFK activity held consistent for the first 40 min postmortem and decreased at 12 hr. Our results show that the PFK loses as much as 75% of its initial activity by 60 min postmortem and this loss may mark the initial stages of arresting postmortem glycolysis.

Curiously, Scopes (1974b) showed PFK maintained activity throughout the postmortem period. Specifically, he drew his conclusion from the ability of his system to achieve a pH of 5.35 at 37 ºC. While his *in vitro* system indeed achieved a pH lower than typical skeletal muscle (pH 5.5 – 5.7), he did not measure PFK activity directly. To address this inconsistency, we suggest that during periods of high metabolic demand, greater glycolytic flux through PFK may be possible before it loses activity. This would ultimately result in greater flux through glycolysis and a lower ultimate pH because the enzymes from aldolase to lactate dehydrogenase function to pH 5.0. One example of this phenomenon may lie with the aggressive and sometimes extended metabolism that occurs in pigs with the halothane gene mutation. Halothane pigs have a mutated ryanodine receptor resulting in accelerated postmortem metabolism (reviewed by Scheffler & Gerrard, 2007). Early postmortem energy demand in muscle of these pigs is high and may result in additional flux through PFK. Once past PFK, F-1,6-BP would then be converted to lactate and H^+ (from ATP hydrolysis) as shown in the previous experiment and result in meat with a lower ultimate pH (Figure 2-4). In many, but not all studies, halothane mutant muscle exhibits a lower ultimate pH (Salmi, et al., 2010) and increased lactate late postmortem (Klont & Lambooy, 1995).

The exact cause for the decrease in PFK activity postmortem *in vivo* is unknown. PFK is temperature and pH labile. As temperature declines from 20 °C to 3 °C, PFK loses as much as 97% of its activity (Bock & Frieden, 1974). Likewise, PFK activity decreases as pH declines (Bock & Frieden, 1976). In both cases, inactivation partially results from the enzyme dissociating from its native tetrameric form to a less active dimeric form (Uyeda, 2006). Lactate accumulation also encourages a similar type of dissociation (Costa-Leite, et al.,

2007). To prevent the dissociation, PFK binds to filamentous actin *in vitro* (Roberts & Somero, 1987). This association also occurs under hypoxic conditions in cancer cells that preferentially ferment glucose to lactate (Gatenby & Gillies, 2004; Vander Heiden, Cantley, & Thompson, 2009). Once bound, glycolytic efficiency increases and PFK is less affected by its inhibitors including lactate (Costa-Leite, et al., 2007). While PFK binding to actin is a protective mechanism against reducing PFK activity antemortem (Roberts, et al., 1987), additional postmortem pH decline likely reduces PFK activity to a point where it arrests postmortem glycolysis.

PFK activity comparison between species

To further test PFK's role in dictating the extent of postmortem metabolism, muscle samples were taken from different species and varied fiber types to determine if low pH induced a similar inactivation. These muscles included chicken *pectoralis major* (glycolytic tissue), beef *semimembranosus*, pork *semitendinosus* (oxidative tissue) and pork LTL. Enzyme activity was evaluated at pH 6.5 and 5.6 with and without 0.15 mM AMP (Figure 2-6). In all cases except the pork LTL, added AMP at pH 6.5 increased ($P < 0.004$) PFK activity. This finding is not surprising given AMP's known status as a potent PFK activator (Berg, Tymoczko, & Stryer, 2007).

Maximal activity (pH 6.5, +AMP) per gram muscle also differed ($P < 0.05$) between species and is due to glycolytic enzyme abundance between muscles of different fiber type (Briand, Talmant, Briand, Monin, & Durand, 1981; Spamer & Pette, 1977). White, glycolytic muscles (chicken *pectoralis major* and pork LTL) contain a greater abundance of glycolytic

enzymes compared to red, oxidative muscles (beef *semimembranosus* and pork *semitendinosus*) (reviewed by Klont, Brocks, & Eikelenboom, 1998).

Despite differences in maximal PFK activity, all species produced a consistent response when tested at pH 5.6 with or without AMP. Essentially, low pH inactivated PFK and AMP is unable to override the inactivation in pork, beef and chicken. Therefore, these data suggest PFK may partially explain the consistency in ultimate pH values across meat of different species typically ranging from 5.5-5.9 (reviewed by England, et al., 2013).

The role of temperature in PFK inactivation

In vitro glycolysis and glycogenolysis were tested at temperatures normally found early postmortem (33, 36, 39 and 42 °C) for 240 min to determine its effect on PFK activity and pH decline. The pH declined with time across all temperatures (Table 2-2A). At 120, 180 and 240 min, the pH of the 42 °C treatment was lower ($P \leq 0.007$) than all other treatments. Similarly, PFK activity of the 42 °C treatment was lower ($P \leq 0.012$) than the 33 °C treatment at the same time-points (Table 2-2B). No activity differences were detected between the 39 or 42 °C at any time-point.

While these results show that maintaining an elevated temperature accelerated PFK activity loss, the activity decline is not likely to be a direct function of temperature alone. Rather, high muscle temperature (i.e. 42 ^{o}C) accelerates postmortem muscle pH decline (Greaser, 1986; Rosenvold & Andersen, 2003; Scheffler & Gerrard, 2007; Solomon, Van Laack, & Eastridge, 1998). These data combined with the previous data and studies by Rhoades et al. (2005) and Werner et al. (2010) lead us to conclude that PFK is extremely susceptible to postmortem pH decline.

Conclusions

These findings expand our collective understanding of how the rate and extent of postmortem metabolism are controlled (Figure 2-7). The rate of pH decline is clearly dictated by ATPase activity (Bowker, et al., 2004; Bowker, et al., 2004; Hamm, 1977; Newbold & Scopes, 1971a, 1971b; Scopes, 1974a, 1974b), while the extent of metabolism or ultimate pH is dictated the amount of muscle glycogen content, as long as levels fall between 0 – 53 μmol / g (Henckel, et al., 2002; Immonen & Puolanne, 2000). In muscles containing glycogen above this amount, we suggest that the loss of PFK activity brackets pH into a normal range of values observed across most meat species. Further, we believe that a rapid glycolysis could lead to meat with a lower ultimate pH by allowing greater amounts of substrate to pass PFK. Additional variation around the ultimate pH of pork (i.e. pH 5.5 – 5.7), or the ability to breach the 'normal' remains to be explained.

Acknowledgments

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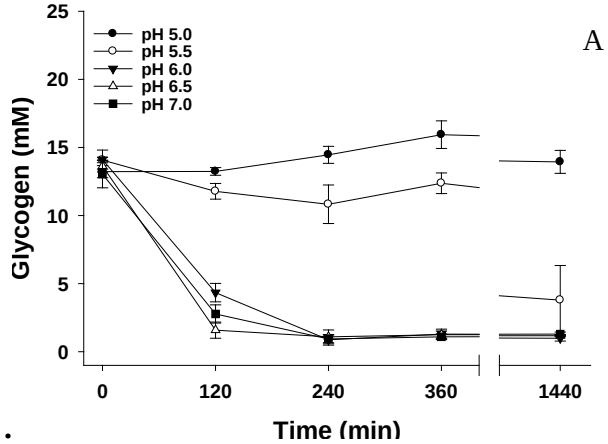
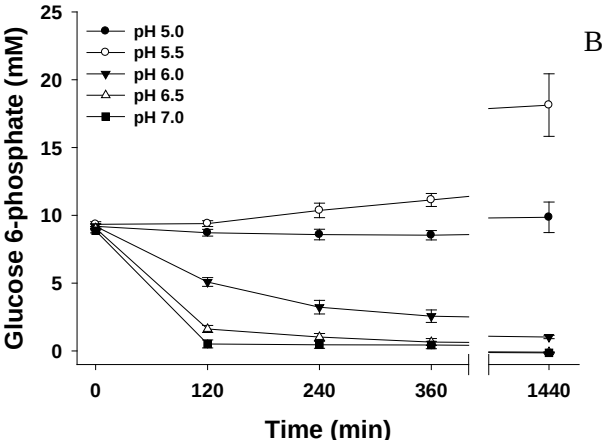




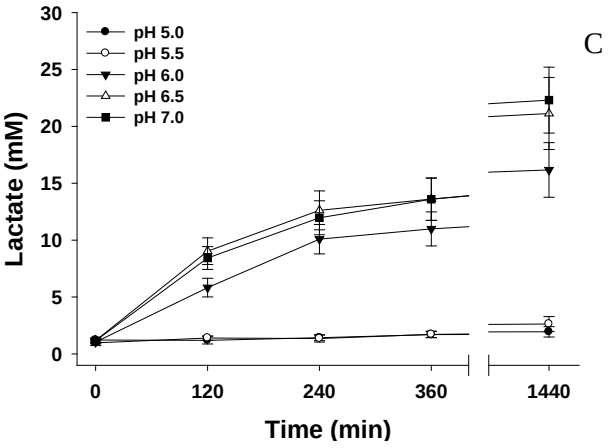


Figure 2-1. Mean glycogen (A), glucose 6-phosphate (B), and lactate (C) concentration (mM) of the reaction buffer and longissimus muscle at buffered pH. Data are mean ± SE.

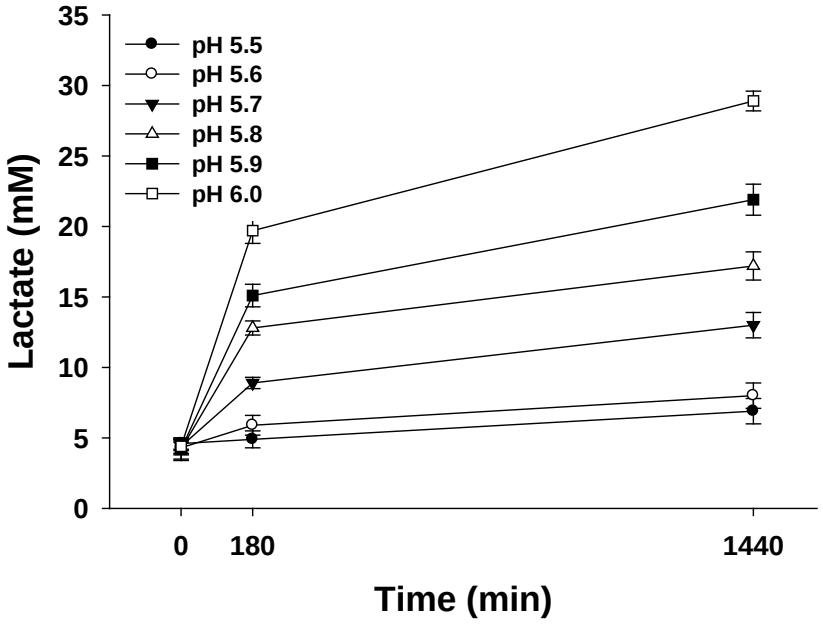


Figure 2-2. Lactate concentration (mM) of the reaction buffer and longissimus muscle at buffered pH. Data are mean ± SE.

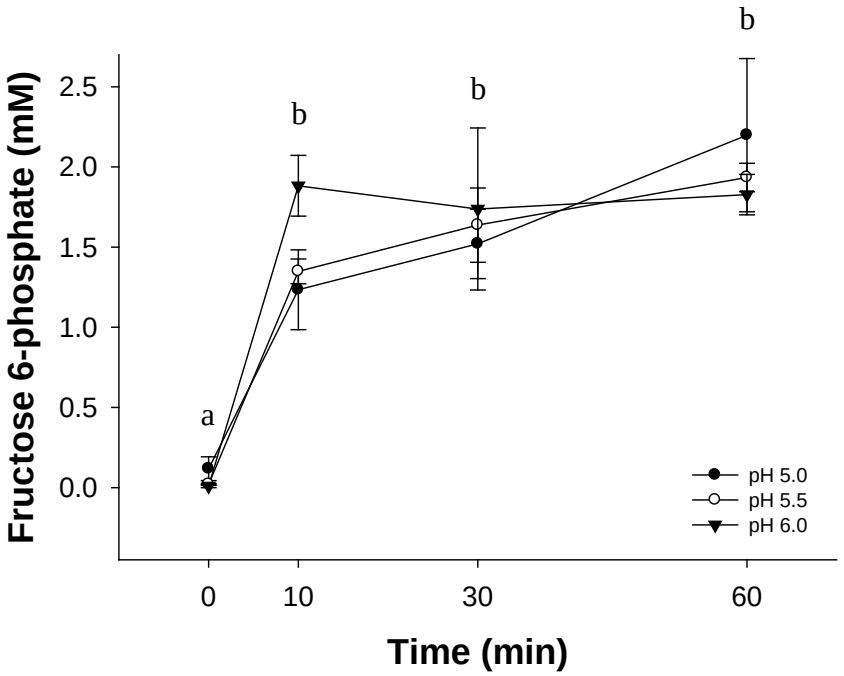


Figure 2-3. Fructose 6-phosphate concentration (mM) of the phosphoglucoisomerase buffer and longissimus muscle at pH 5.0, 5.5 and 6.0.Data are mean ± SE. Time-points without a common superscript (a,b) are different ($P \leq 0.03$) for all treatments.

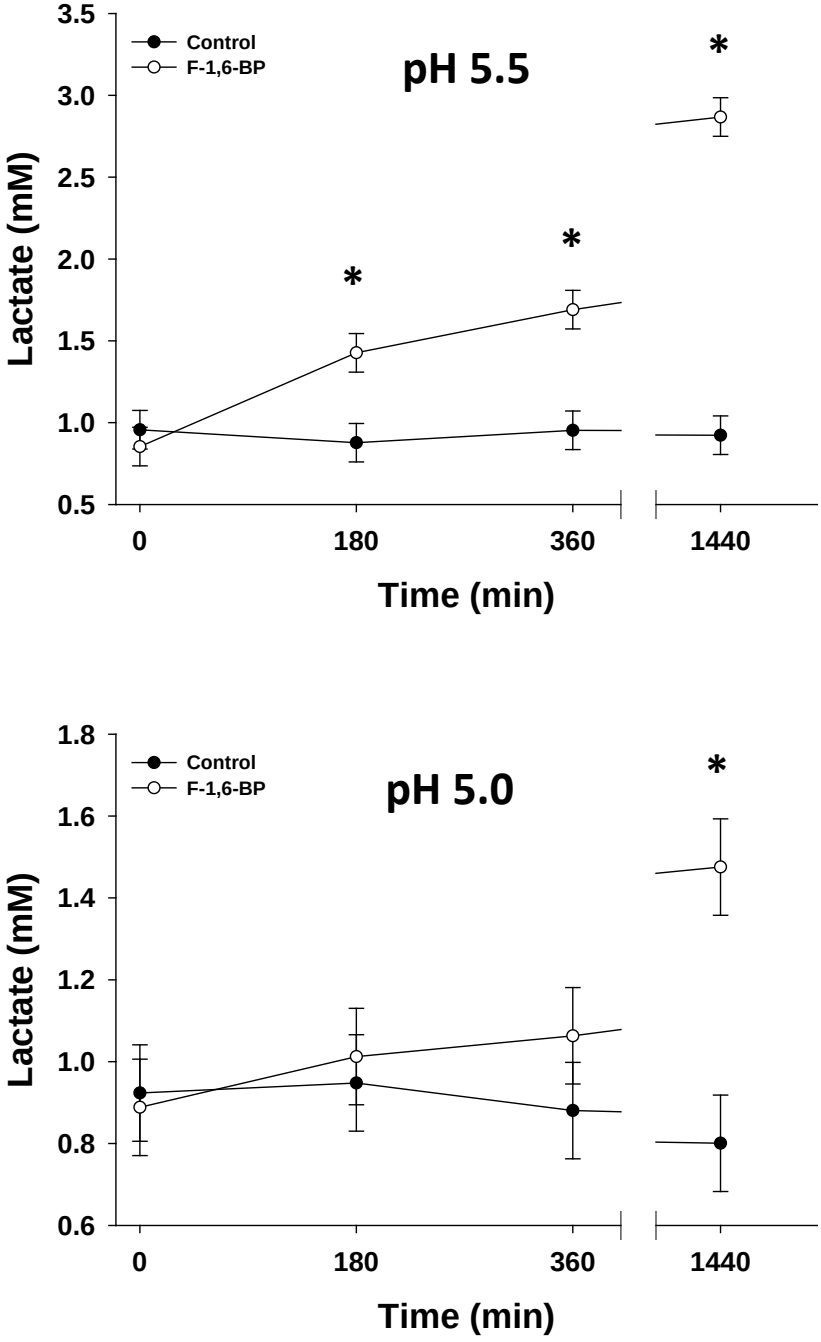
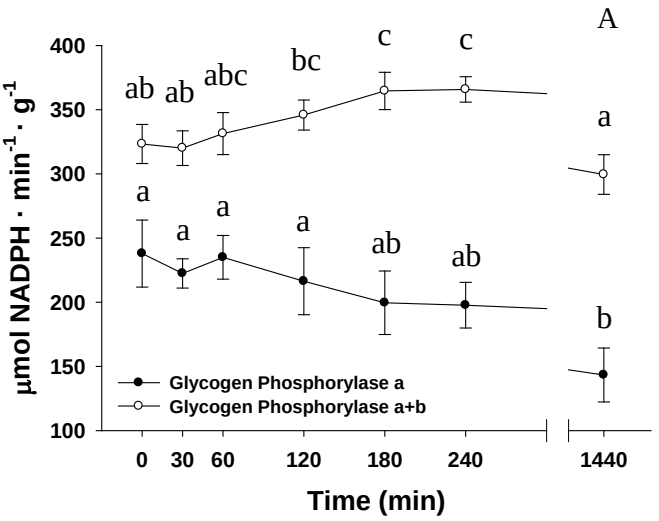
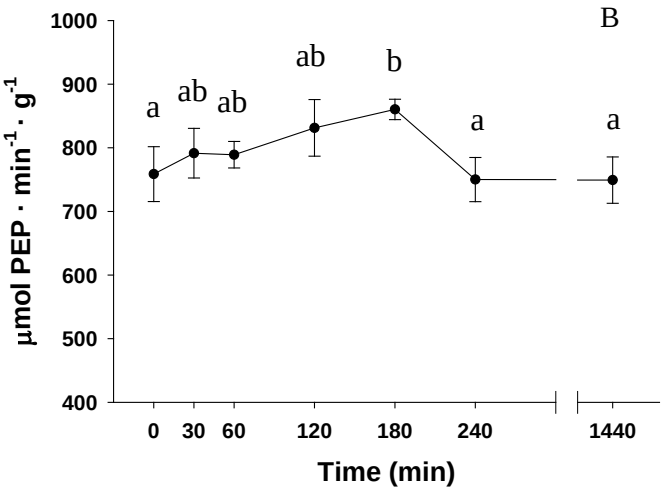
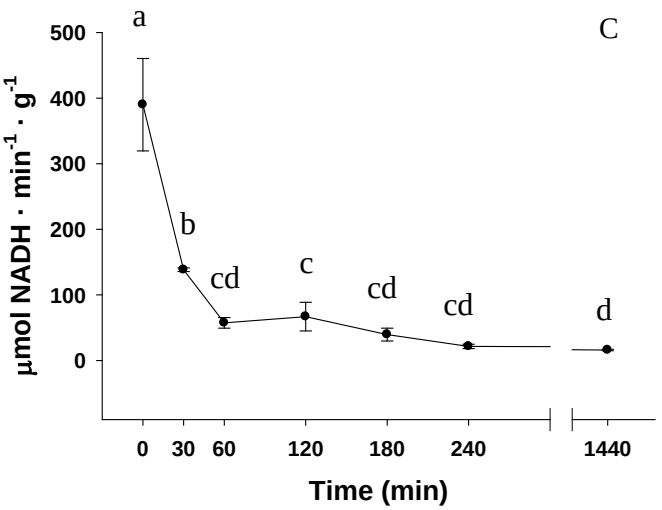


Figure 2-4. Lactate concentration (mM) of the reaction buffer and longissimus muscle at pH 5.5 and 5.0. Treatments are control or added 4 mM fructose 1,6-bisphosphate (F-1,6-BP). Data are mean ± SE. *P < 0.01.


A
µmol NADPH · min-1 · g-1
400
350
300
250
200
150
100
ab
ab
abc
bc
c
c
a
a
a
a
ab
ab
a
b
Glycogen Phosphorylase a
Glycogen Phosphorylase a+b
0
30
60
120
180
240
1440
Time (min)


B
µmol PEP · min-1 · g-1
1000
900
800
700
600
500
400
a
ab
ab
ab
b
a
a
0
30
60
120
180
240
1440
Time (min)


C
µmol NADH · min-1 · g-1
500
400
300
200
100
0
a
b
cd
c
cd
cd
d
0
30
60
120
180
240
1440
Time (min)

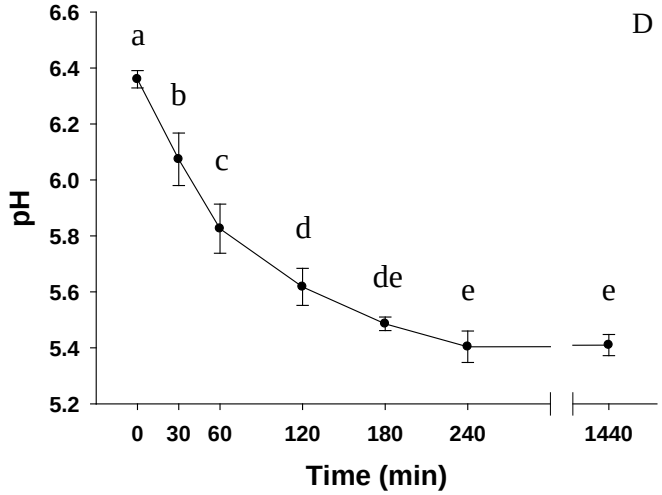


Figure 2-5. Postmortem enzymatic activity of glycogen phosphorylase (A), pyruvate kinase (B), phosphofructokinase (C) and muscle pH (D) from five pigs. Data are mean ± SE. Means within an individual enzyme or pH without a common superscript (a,b,c,d) are different.

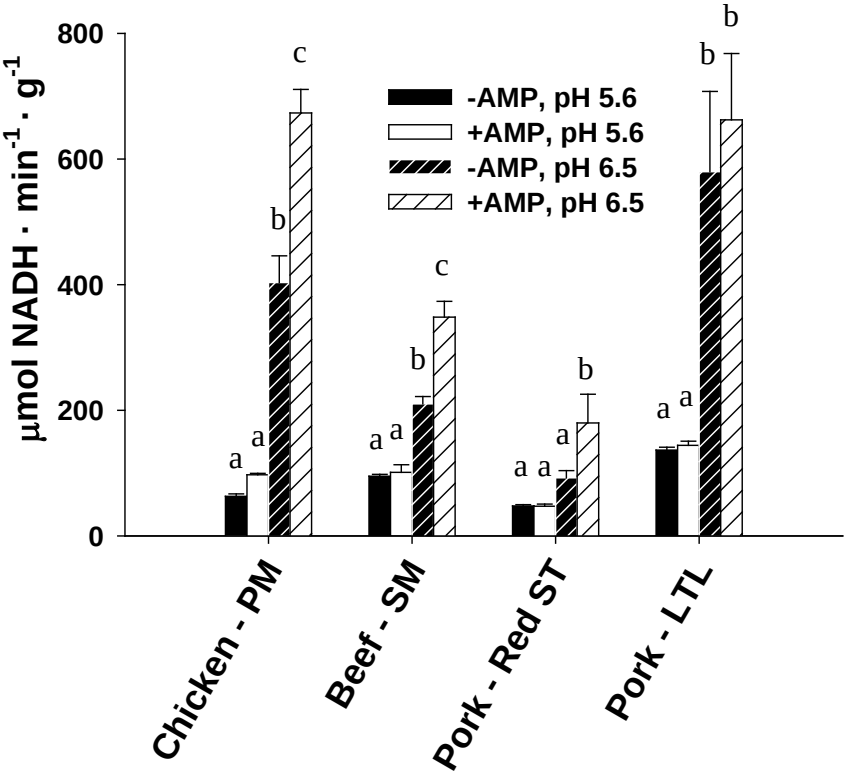


Figure 2-6. Phosphofructokinase activity comparison between pH, AMP and species between chicken pectoralis major (3 animals; PM), beef semimembranosus (3 animals; SM), pork semitendinosus (3 animals; ST) and pork longissimus muscle (3 animals; LTL). Data are mean ± SE. The means without a common superscript (a, b, c) within a species/muscle grouping differ ($P < 0.05$).

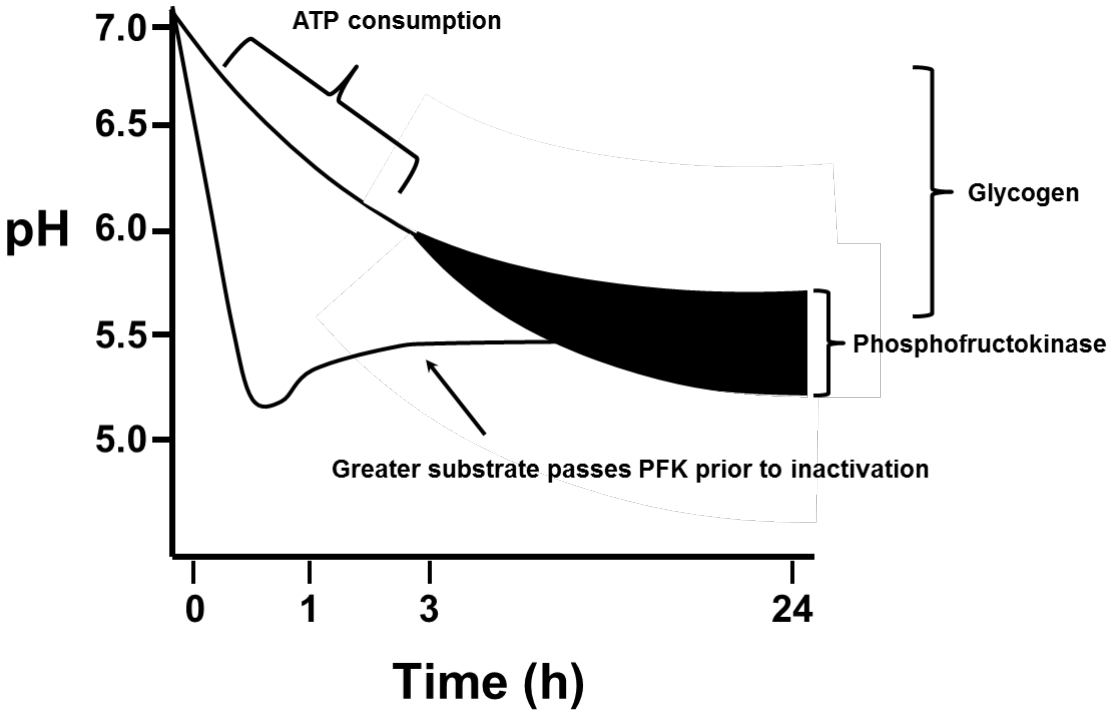


Figure 2-7. Working model of the factors controlling the rate and extent of postmortem metabolism. References detailing the role of ATP consumption and glycogen are included in the text.

Table 2-1. Sum total carbohydrate (glycogen + glucose + sugar phosphates; mM) of the reaction buffer and longissimus muscle. Data are mean ± SE. Means without a common superscript (a, b) differ ($P < 0.05$).

		Time (hr)	
		0	24
pH Treatment	5.0	22.5 ± 0.3 [a]	23.4 ± 0.3 [a]
	5.5	23.4 ± 0.4 [a]	21.8 ± 0.4 [a]
	6.0	23.3 ± 0.4 [a]	3.5 ± 0.4 [b]
	6.5	22.7 ± 0.2 [a]	2.4 ± 0.3 [b]
	7.0	21.9 ± 0.4 [a]	2.2 ± 0.4 [b]

Table 2-2. Muscle homogenate and reaction buffer pH (A) and phosphofructokinase activity (B; μmol NADH * min^{-1} * g^{-1}) held at constant temperatures. Data are mean ± SE. Means within a row without a common superscript (a, b, c) differ ($P < 0.05$).

		Temperature (°C)				**A**
		33	36	39	42	
Time (min)	0	6.76 ± 0.04^{a}	6.81 ± 0.03^{a}	6.81 ± 0.04^{a}	6.71 ± 0.05^{a}	
	30	6.19 ± 0.02^{b}	6.32 ± 0.01^{a}	6.35 ± 0.03^{a}	6.16 ± 0.04^{b}	
	60	6.11 ± 0.02^{bc}	6.17 ± 0.01^{ab}	6.22 ± 0.03^{a}	6.07 ± 0.05^{c}	
	120	5.98 ± 0.03^{a}	6.03 ± 0.03^{a}	6.00 ± 0.07^{a}	5.80 ± 0.07^{b}	
	180	5.88 ± 0.04^{a}	5.89 ± 0.04^{a}	5.81 ± 0.05^{a}	5.68 ± 0.04^{b}	
	240	5.79 ± 0.03^{a}	5.76 ± 0.05^{a}	5.76 ± 0.03^{a}	5.62 ± 0.01^{b}	

		Temperature (°C)				**B**
		33	36	39	42	
Time (min)	0	180.3 ± 22.9^{a}	168.3 ± 21.9^{a}	169.4 ± 16.5^{a}	171.9 ± 27.5^{a}	
	30	88.3 ± 19.5^{a}	85.4 ± 14.4^{a}	87.8 ± 11.0^{a}	77.1 ± 13.1^{a}	
	60	42.3 ± 5.3^{b}	72.0 ± 11.4^{a}	50.5 ± 9.7^{ab}	44.7 ± 3.4^{b}	
	120	61.8 ± 9.9^{a}	48.6 ± 8.3^{ab}	38.8 ± 3.0^{b}	33.0 ± 6.9^{b}	
	180	61.8 ± 13.6^{a}	43.4 ± 6.4^{ab}	25.2 ± 5.0^{b}	22.6 ± 5.5^{b}	
	240	45.7 ± 7.4^{a}	44.1 ± 8.1^{a}	26.6 ± 6.2^{ab}	9.3 ± 4.7^{b}	

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Chapter 3 – Altered AMP deaminase activity may extend postmortem glycolysis

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Abstract

Postmortem energy metabolism drives hydrogen accumulation in muscle and results in a fairly constant ultimate pH. Extended glycolysis results in adverse pork quality and may be possible with greater adenonucleotide availability postmortem. We hypothesized that slowing adenonucleotide removal by reducing AMP deaminase activity would extend glycolysis and lower the ultimate pH of muscle. *Longissimus muscle* samples were incorporated into an *in vitro* system that mimics postmortem glycolysis with or without pentostatin, an AMP deaminase inhibitor. Pentostatin lowered ultimate pH and increased lactate and glucose 6-phosphate with time. Based on these results and that AMPK $\gamma3^{R200Q}$ mutated pigs (RN^-) produce low ultimate pH pork, we hypothesized AMP deaminase abundance and activity would be lower in RN^- muscle than wild-type. RN^- muscle contained lower AMP deaminase abundance and activity. These data show that altering

adenonucleotide availability postmortem can extend postmortem pH decline and suggest that AMP deaminase activity may, in part, contribute to the low ultimate pH observed in RN^- pork.

Introduction

During the conversion of muscle to meat, both the rate and the extent of pH decline dictate pork quality. While the rate of pH decline is controlled by ATPase activity (Newbold & Scopes, 1971a, 1971b; Scopes, 1974a, 1974b), the mechanism responsible for determining ultimate pH is not fully explained. We recently reported that phosphofructokinase is responsible for bracketing ultimate pH into a fairly consistent range near pH 5.6 (England, Matarneh, Scheffler, Wachet, & Gerrard, 2014). However, phosphofructokinase inactivation may not explain the relative ultimate pH variability (pH 5.4 – 5.8) between pigs from a genetically similar background. Above an ultimate pH of 5.6, muscle glycogen determines ultimate pH (Henckel, Karlsson, Jensen, Oksbjerg, & Petersen, 2002; Immonen & Puolanne, 2000). However, the biochemical mechanism responsible for pork with an ultimate pH below 5.6 is unknown.

During postmortem metabolism, skeletal muscle attempts to maintain cellular homeostasis and ATP levels. ATP is produced by rephosphorylating ADP using creatine kinase, phosphoglycerate kinase, pyruvate kinase and adenylate kinase. Adenylate kinase (EC 2.7.4.3) converts two ADP molecules to one ATP and one AMP molecule (Pearson, 1971). Once created by adenylate kinase, AMP is converted to IMP by AMP deaminase (EC 3.5.4.6) (Korzeniewski, 2006). Given the importance of postmortem ATP generation

and the equilibrium constant of the adenylate kinase reaction, the removal of AMP drives the reaction to the right to increase ATP formation.

However, AMP deaminase's action also lowers the available adenonucleotide pool because IMP is an end product metabolite and unable to contribute to ATP formation (Hamm, 1977). The complete conversion of adenonucleotides to IMP coincides with the completion of postmortem glycolysis (Bendall, 1973; Dalrymple & Hamm, 1975; Greaser, 2001). Therefore, AMP deaminase activity may establish the termination of postmortem glycolysis and the ultimate pH of meat. AMP deaminase activity is positively correlated to ultimate pH in both pigs and chickens (El Rammouz, Berri, Le Bihan-Duval, Babile, & Fernandez, 2004; van Laack, Yang, & Spencer, 2001). In addition, when increasing levels of AMP deaminase were included in an *in vitro* buffer system, the termination of anaerobic glycolysis occurred earlier and resulted in an elevated ultimate pH (Scopes, 1974b). Therefore, the opposite may also be true. To that end, we hypothesized that reducing AMP deaminase activity will retain the availability of adenonucleotides to extend glycolysis and lower ultimate pH.

Materials and methods

Animals

Market weight pigs (100 – 125 kg) were harvested in the Virginia Tech Meat Center using accepted commercial processing procedures. Following exsanguination, samples were excised from the *longissimus muscle* at 0, 30, 60, 120, 180 and 1440 min postmortem, snap frozen in liquid nitrogen and stored at -80 ^{o}C.

In Vitro Buffer System

Wild-type porcine *longissimus muscle* samples (n = 8) were powdered in liquid nitrogen and homogenized at a 1:10 ratio (wt/vol) with a reaction buffer containing 10 mM Na_2HPO_4 (pH 7.4), 5 mM $MgCl_2$, 60 mM KCl, 5 mM ATP, 0.5 mM NAD+, 40 mM glycogen, 0.5 mM ADP, 25 mM carnosine, 30 mM creatine, and 10 mM sodium acetate and incubated at 25 ^oC (England, et al., 2014). The pentostatin treatment contained 150 μM pentostatin (Tocris Bioscience, Minneapolis, MN). Aliquots were removed from the reaction buffer for pH and metabolite analysis at 0, 30, 120, 240 and 1440 min.

pH Analysis

Measurement of pH was conducted according to Bendall (1973) with a slight modification. Four volumes of homogenate were transferred to a new tube and one volume of 25 mM sodium iodoacetate and 750 mM KCl (pH 7.0) was added. Samples were equilibrated to 25 ^oC, centrifuged and measured immediately thereafter using a Thermo Scientific Orion Ross Ultra Semi-Micro glass electrode (Thermo Scientific, Pittsburgh, PA).

Metabolite Analysis

Aliquots were removed from the homogenate for glycogen analysis and mixed with an equal volume of 2.5 M HCl, heated at 90 ^oC for 2 h, centrifuged, and the resulting supernatant was neutralized with 1.25 M KOH (Bergmeyer, 1984). Samples were removed from the homogenate for lactate, glucose 6-phosphate, adenonucleotide and IMP analysis and treated with an equal volume of ice-cold 1 M perchloric acid, centrifuged, and the resulting supernatant was neutralized with 2 M KOH (Bergmeyer, 1984).

Glycogen, lactate, glucose, and glucose 6-phosphate were measured using previous methods (Bergmeyer, 1984) modified for microplate volumes (Hammelman et al., 2003). All reactions occurred in 5 mL borosilicate glass tubes and were analyzed spectrophotometrically at 340 nm in triplicate using 96-well microplates. ATP, ADP, AMP and IMP were separated with an HP Agilent 1100 (Agilent Technologies, Santa Clara, CA) using an Accucore C18 2.6 μm 50 mm x 4.6 mm column (Thermo Scientific, Pittsburgh, PA) and detected at 254 nm with gradient separation (Bernocchi, et al., 1994; Williams, Vidt, & Rinehart, 2008).

AMP Deaminase Activity

AMP deaminase activity was measured from postmortem muscle at 0, 30, 60, 120 and 1440 min (Raggi & Ranieri-Raggi, 1987). Frozen muscle was powdered in liquid nitrogen and homogenized at a 4% (wt/vol) suspension with 90 mM K_2HPO_4 (pH 6.5), 180 mM KCl, and 0.1 mM dithiothreitol. Aliquots of the homogenate were added to the reaction buffer containing 200 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 150 mM KCl, and 10 mM AMP at pH 6.5 unless otherwise specified. Time-course samples were removed and processed for HPLC analysis as described previously. AMP deaminase activity was determined by the decrease of AMP with time and reported as μmol AMP * min^{-1} * mg^{-1}.

Gel electrophoresis and Transfer

Muscle tissue was powdered in liquid nitrogen and the proteins solubilized according to Warren, Krzesinski, & Greaser (2003) (Warren, Krzesinski, & Greaser, 2003) (Warren, Krzesinski, & Greaser, 2003) (Warren, Krzesinski, & Greaser, 2003) (Warren, Krzesinski,

& Greaser, 2003) (Warren, Krzesinski, & Greaser, 2003). Solubilized proteins from the *longissimus muscle* (0 min postmortem) of wild-type and RN^- pigs (n = 8 for each genotype) were separated by SDS-PAGE (10%), transferred to nitrocellulose membranes and immunoblotted with primary antibodies specific for AMP deaminase (Abcam, Cambridge, MA) and actin (Developmental Studies Hybridoma Bank, Iowa City, IA). Bands were visualized with IRDye fluorescent secondary antibodies (LI-COR Biosciences, Lincoln, NE) and quantified using LI-COR Biosciences Odyssey imaging system and software.

Statistical Analysis

Data are presented as means ± SE. All data were analyzed with a mixed model in JMP (SAS Institute Inc., Cary, NC). Individual animals were recognized as an experimental unit and time-course data were analyzed with a split-plot design and all differences are presented within time. Single exponential decay curves to an arbitrary value (ultimate pH) were derived to determine the exponential decay constant using Sigma Plot (Systat Software, Inc., San Jose, CA) to determine the mean rate of pH decline. The least squares means were evaluated using a Student's t-test and considered significant at $P \leq 0.05$.

Results and discussion

Pentostatin In Vitro

We utilized 2-deoxycoformycin (pentostatin) to slow adenonucleotide removal in an *in vitro* postmortem glycolysis model (England et al., 2014). Pentostatin is a purine analog

that inhibits AMP deaminase and adenosine deaminase (EC 3.5.4.4) (Agarwal & Parks Jr, 1977). Adenosine deaminase converts adenosine to inosine and its inhibition likely plays little role in altering postmortem glycolysis. The *in vitro* buffer system contained 150 μM pentostatin because 60 μM provided 94% relative inhibition of rabbit muscle AMP deaminase (Fishbein, 1979) and porcine muscle contains four to five times greater AMP deaminase activity than rabbit muscle (Fishbein, Davis, & Foellmer, 1993).

Reducing AMP deaminase activity decreased adenonucleotide conversion to IMP (Table 3-1). At 30 min, the pentostatin treatment contained greater ATP, greater ADP and lower IMP ($P < 0.0001$). At 120 min, AMP was greater ($P = 0.0254$) in the pentostatin treatment. Combined, the greater adenonucleotides and lower IMP indicate that AMP deaminase activity was reduced and showed the efficacy of pentostatin in our system. However, AMP was elevated at 0 min in both treatments compared to later time points even though AMP was not added to the buffer system. It is unclear why AMP is highest at 0 min, but the same phenomenon was evident during other studies in our laboratory using this *in vitro* system (data not shown). It is possible that the muscle tissue incorporated in the buffer system contained elevated AMP, though postmortem porcine muscle tissue only contains between 0.05 – 0.20 μmol/g AMP (Kastenschmidt, Hoekstra, & Briskey, 1968). Given we dilute tissue 1:10 in our system, the measured AMP likely develops because AMP deaminase requires tissue acidification (Dudley & Terjung, 1985) and/or increased AMP for activation (Ronca-Testoni, Raggi, & Ronca, 1970; Smiley, Berry, & Suelter, 1967). Despite this anomaly, the reduction in AMP deaminase activity slowed the removal of adenonucleotides and resulted in hastened and extended glycolysis.

Pentostatin enhanced glycogen catabolism with lower residual glycogen at 240 and 1440 min ($P \leq 0.0013$; Figure 3-1). The increased utilization of glycogen also resulted in greater lactate formation at 30, 120, 240 and 1440 min ($P \leq 0.02$; Figure 3-2). Greater lactate production is indicative of extended glycolysis which yields a greater total number of ATP molecules by the distal elements of glycolysis. Because hydrogens produced from ATP hydrolysis ($ATP \rightarrow ADP + H^+$) are responsible for muscle acidification postmortem (Hamm, 1977), extended postmortem glycolysis lowers ultimate pH. To that end, the pentostatin produced lower pH at 30, 120, 240 and 1440 min ($P \leq 0.0082$; Figure 3-3). Specifically, reducing AMP deaminase activity extended ultimate pH from 5.59 ± 0.02 in the control group to 5.42 ± 0.01 in the treated group. Combined, these metabolite and pH data show that reducing *in vitro* AMP deaminase activity extended anaerobic glycolysis.

Reducing AMP deaminase activity also accelerated the rate of pH decline. By fitting the data to a single exponential decay curve to an arbitrary value (ultimate pH) (Figure 3-4A), the exponential decay constant for pH declines containing pentostatin were greater ($P = 0.0177$) than controls (Figure 3-4B). The exact reason underlying the accelerated pH decline is unclear, but greater availability of ADP for rephosphorylation may be a contributor. Alternatively, elevated AMP may have activated phosphofructokinase and/or AMPK. Activated AMPK can phosphorylate and activate phosphofructokinase-2. Activation of phosphofructokinase-2 produces fructose-2,6-bisphosphate, a potent activator of phosphofructokinase. Production of fructose-2,6-bisphosphate was not measured, but clearly accelerates glycolysis (Shen & Du, 2005; Shen, et al., 2006; Shen, Underwood, Means, McCormick, & Du, 2007).

Because pentostatin perturbs glycolysis in this *in vitro* system, it is important to address our results in how they may provide insight into mechanisms responsible for pale, soft, and exudative (PSE) meat or acid meat. Historically, PSE refers to inferior meat produced by the rapid acidification of postmortem muscle at elevated temperatures normally associated with a mutation in the porcine stress syndrome gene (i.e. Halothane pigs). Acid meat refers to low ultimate pH and is often times associated with RN^- pigs (Milan, et al., 1996). In both cases, the meat produced has a pale color, soft texture and lower water holding capacity (Briskey, 1964). Essentially, the classically measured fresh meat quality characteristics (color, texture, water holding capacity) seem quite similar for both genotypes. Yet, limited information is available on what functional properties, if any, differ between PSE and acid meat. This idea is particularly intriguing given some studies have shown that halothane pigs can produce both a rapid postmortem metabolism and lower ultimate pH (Salmi, et al., 2010). When we determined the meat quality characteristics from double mutant pigs (Halothane x RN^-), an additive effect was noted for b* (yellowness) and drip loss, and the ultimate pH approached significance (Copenhafer, Richert, Schinckel, Grant, & Gerrard, 2006). These data suggest early and late postmortem effects may be separate events and the hydrogen production, albeit from ATP hydrolysis may be additive. Understanding changes in the functional properties between pork produced by these two genotypes may be useful in providing further insight into the biochemical basis controlling these two fresh meat quality-driving mechanisms.

Even so, the aforementioned results help clarify the development of low ultimate pH pork. In this study, we show that addition of pentostatin to our *in vitro* system extends anaerobic glycolysis. We recently reported that during 'normal' postmortem metabolism,

phosphofructokinase activity begins to slow just below 6.0, terminating glycolysis and bracketing the ultimate pH around 5.6 $\pm$ 0.2 (England, et al., 2014). In that study we also showed that glycolytic metabolites traversing phosphofructokinase ultimately produce lactate to extend glycolysis provided the environmental pH is not below 5.0. When considered in totality, pH decline beyond pH 5.6 may occur when phosphofructokinase activity is maintained near pH 6.0 by elevated AMP that allows additional flux through glycolysis and lower ultimate pH. Vetharaniam, Thomson, Devine, & Daly (2010) corroborated this claim when they modeled anaerobic postmortem energy metabolism and determined AMP deaminase activity stopped in conjunction to a sharp decline in glycolytic activity.

Curiously, glucose 6-phosphate was greater in the pentostatin treatment at 240 and 1440 min ($P < 0.0001$; Figure 3-5). This was somewhat surprising but when combined with the lower pH data resembled RN^- pig muscle that produces a lower pH and greater glucose 6-phosphate by 24 hr postmortem (Copenhafer, et al., 2006; Monin & Sellier, 1985). Why increased glucose 6-phosphate is produced late postmortem in muscle is not well known. Enzymes responsible for converting glycogen to glucose 6-phosphate are glycogen phosphorylase and phosphoglucomutase. Elevated AMP, similar to that caused by the pentostatin treatment, enhances glycogen phosphorylase *b* activity which should result in the increased glucose 6-phosphate late postmortem. To that end, we determined adenonucleotides and IMP levels late postmortem (180 min) when glucose 6-phosphate is known to be different between wild-type and RN^- pigs. Elevated glucose 6-phosphate and AMP occurred simultaneously (Table 3-2). When combined, the pH and metabolite profile

produced by the pentostatin treatment and this *in vivo* metabolite profile suggested AMP deaminase activity and/or abundance may differ between wild type and RN^- pig muscle

RN^- pigs have a single nucleotide substitution in the PRKAG3 gene which encodes for the muscle specific γ3 regulatory subunit of AMPK (Milan, et al., 2000). This substitution causes AMPK to be constitutively active. These animals deposit higher muscle glycogen and produce inferior meat with an ultimate pH near 5.3 (Copenhafer, et al., 2006; Enfält, Lundström, Hansson, Johansen, & Nyström, 1997). Yet, the increased glycolytic potential does not cause the low ultimate pH (Scheffler, Scheffler, Kasten, Sosnicki, & Gerrard, 2013). No satisfactory explanation has been reported to explain the low ultimate pH of RN^- pork. While the *longissimus muscle* of mutant pigs contains similar ATP levels as wild-type pigs, they have elevated phosphocreatine in their muscle at harvest (Copenhafer, et al., 2006). Because phosphocreatine is utilized quickly to rephosphorylate ADP by creatine kinase (EC 2.7.3.2) (Robergs, Ghiasvand, & Parker, 2004), it could be argued that RN^- pigs contain a greater total ATP pool early postmortem. In addition, RN^- *longissimus muscle* is more oxidative with a lower proportion of IIb fibers than wild-type animals (Lebret, et al., 1999; Park, Gunawan, Scheffler, Grant, & Gerrard, 2009). Because red, oxidative muscles tend to exhibit lower AMP deaminase activity than white, glycolytic muscles in many species (Fishbein, et al., 1993) and because AMP was higher at 180 min postmortem (Table 3-2), we hypothesized that RN^- muscle contained lower AMP deaminase activity and abundance. If so, it may extend the availability of adenonucleotides and allow for extended postmortem glycolysis which in turn may explain the low ultimate pH of RN^- pork.

AMP Deaminase In Vivo

We initially compared AMP deaminase activity between wild-type and RN^- *longissimus muscle* at the optimal pH of 6.5 of rabbit skeletal muscle AMP deaminase (Raggi & Ranieri-Raggi, 1987). The data showed decreased activity in the RN^- pigs. A pH-activity curve was constructed to determine indirectly if this initial activity difference was related to abundance or isoenzyme profile in the RN^- pigs (Figure 3-6). The data showed significant genotype ($P = 0.0002$) and pH ($P < 0.0001$) effects with the RN^- pigs having lower activity at all pH values tested, but no interactions were detected ($P = 0.8340$) suggesting a difference in enzyme abundance. It also showed the pH optimum for porcine AMP deaminase is between pH 6.0 and 6.5. To explain the activity difference, RN^- pig *longissimus muscle* contained less AMP deaminase ($P = 0.03$) protein abundance compared to wild-type animals (Figure 3-7).

AMP deaminase activity was also measured to determine the activity changes that occur during postmortem metabolism. RN^- pig muscle contained lower ($P \leq 0.033$) AMP deaminase activity at 0, 30, 60, and 120 min postmortem (Figure 3-8). The initial difference is likely due to the lower AMP deaminase concentration in the RN^- pigs (Figure 3-7). However, because it was not measured, it is possible the wild-type AMP deaminase was at a greater phosphorylation state and may explain the activity differences. Phosphorylation by protein kinase C activates AMP deaminase (Tovmasian, Hairapetian, Bykova, Severin Jr, & Haroutunian, 1990). However, AMP deaminase was not among the enzymes with different phosphorylation states between wild-type and RN^- pigs (Lametsch, et al., 2011).

AMP deaminase is muscle fiber-type specific with greater enzymatic activity and mRNA expression in white, glycolytic muscles of pigs (Aberle & Merkel, 1968; Fishbein, et al., 1993; Wang, et al., 2008). We investigated AMP deaminase mRNA expression between the genotypes, but no differences were detected (data not shown). Additionally, AMP deaminase activity increases with a decreasing energy charge in muscle (Coffee & Solano, 1977). Presumably, the opposite may also occur. The cellular manipulation of AMP deaminase abundance may function to maintain the availability of adenonucleotides during times of great energy demands on cells, in the case muscle. One of the key defining characteristics of RN^- muscle is the high energy charge. These pig muscles contain increased glycogen and creatine phosphate (Copenhafer, et al., 2006; Lindahl, Henckel, Karlsson, & Andersen, 2006). To our knowledge, no direct molecular connection between AMPK and AMP deaminase has been reported. However, chronic activation of AMPK initiates a number of energy producing and protein expression pathways (Holmes, Kurth-Kraczek, & Winder, 1999; McGee, et al., 2008) and a connection between the two might be possible because AMP levels directly affect AMPK activation.

In conclusion, lower AMP deaminase activity and abundance may contribute to the lower ultimate pH of RN^- pork. We have demonstrated in an *in vitro* system used to mimic postmortem glycolysis and pH decline that slowing AMP deaminase recapitulates that which is observed in RN- muscle, namely a reduction in the ultimate pH and an elevation of glucose 6-phosphate. However, this system results in a more aggressive pH decline early, which has never been documented to occur in muscle of RN^- pigs postmortem. Moreover, to conclude that AMP deaminase is responsible for extended postmortem glycolysis in the RN^-, using our current understanding of postmortem energy metabolism

(Figure 3-9) requires that the mutant animals produce greater lactate than wild-type animals. We have shown previously that 24 hr lactate may or may not be elevated in RN^- pig muscle, yet still have lower pH values at 24 hr (Copenhafer, et al., 2006; Scheffler, et al., 2013). This raises the question of whether the development of acid meat (RN^-) is truly a result of an extended glycolysis or some other mechanism related to altered muscle metabolism in these mutant pigs. If extended glycolysis is responsible for this aberrant meat quality, then AMP deaminase contributes to the process. Otherwise it is simply a result of highly elevated energy charge in the tissue and only associated with the defect.

Conclusions

Overall, these data expand our collective understanding of how ultimate pH is determined. They show that reducing AMP deaminase activity may be capable of extending glycolysis as evident by the lower ultimate pH, greater glycogen catabolism and increased lactate formation. Therefore, AMP deaminase activity may explain, at least in part, the variation in ultimate pH of most meat species. To that end, we have updated our working model (England et al., 2014) of the factors controlling the rate and extent of postmortem metabolism (Figure 3-9). Data from this study may also provide additional selection indices for developing superior pork quality genotypes with higher ultimate pH with improved quality characteristics and/or provide targets capable of predicting pork quality early postmortem. Finally, RN^- pig *longissimus muscle* contains less AMP deaminase activity and abundance than wild-type pigs which may contribute to the lower ultimate pH pork from these animals.

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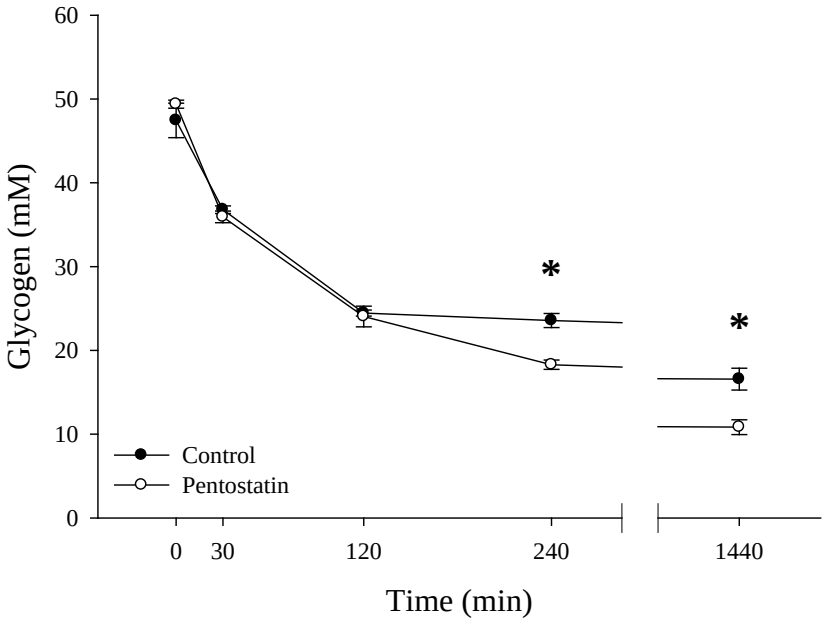

Figure 3-1. Mean glycogen (mM) of longissimus muscle homogenate. Data are mean ± SE. *P < 0.05 within each time-point.

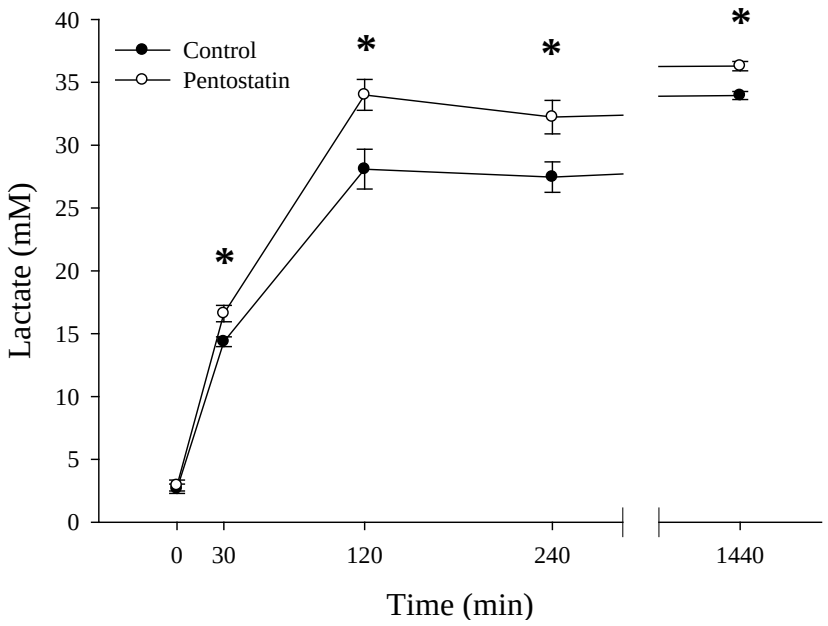

Figure 3-2. Mean lactate (mM) of longissimus muscle homogenate. Data are mean ± SE. *P < 0.05 within each time-point.

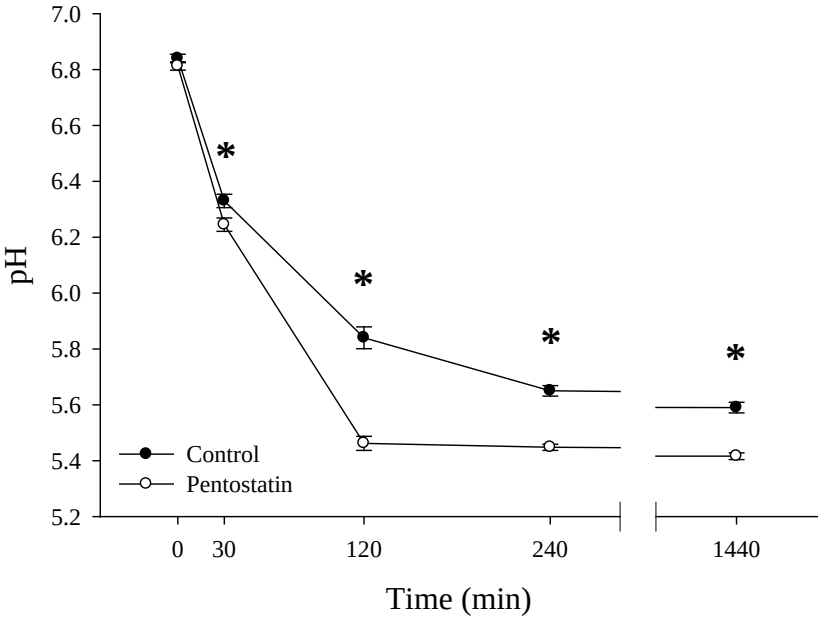

Figure 3-3. Mean pH of longissimus muscle homogenate. Data are mean ± SE. *P < 0.05 within each time-point.

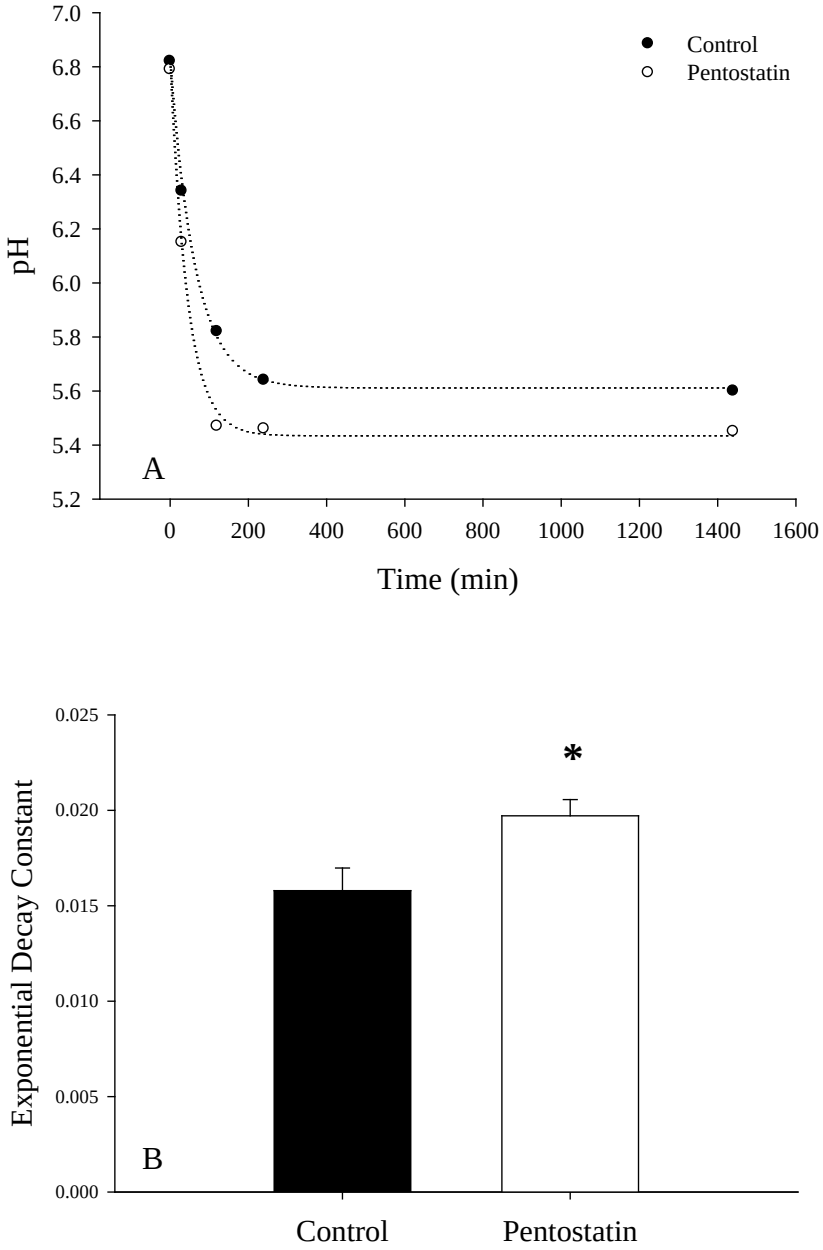

Figure 3-4. (A) Representative graph derived from fitting a non-linear single exponential decay to ultimate pH to determine exponential decay constants. (B) Mean rate of exponential decay constants for pH decline. Data are mean ± SE. *P < 0.05

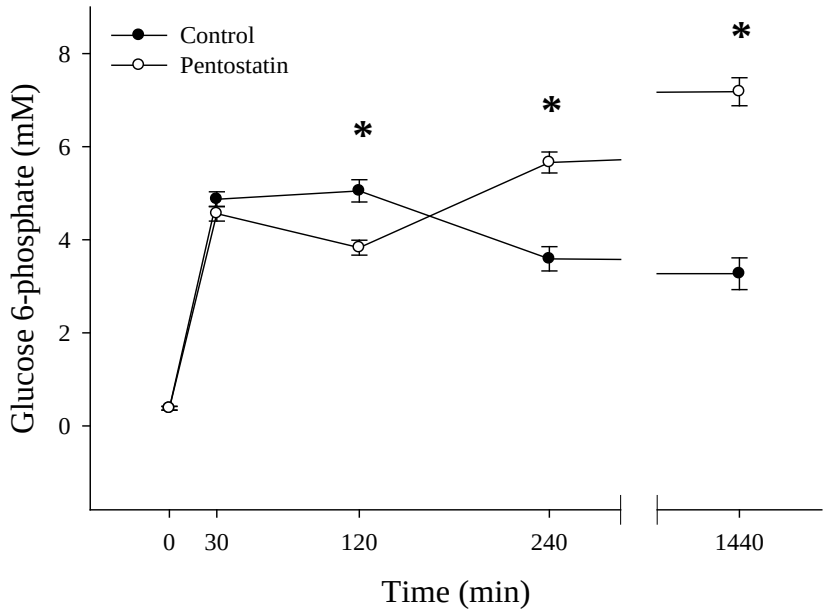

Figure 3-5. Mean glucose 6-phosphate (mM) of longissimus muscle homogenate. Data are mean ± SE. *P < 0.05 within each time-point.

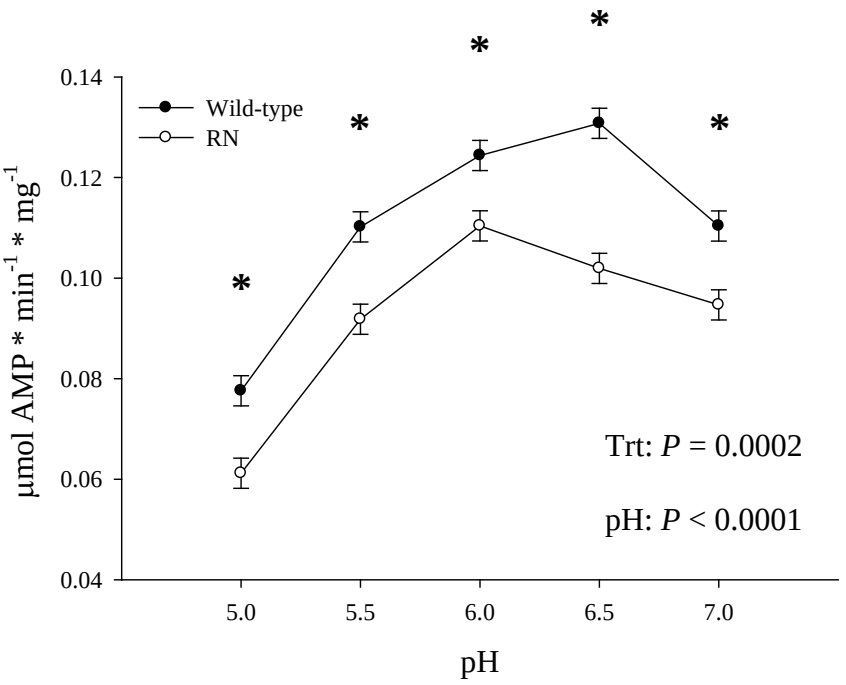

Figure 3-6. Mean AMP deaminase activity of wild-type or AMPK $\gamma3^{R200Q}$ (RN) pigs. Data are mean ± pooled SE. *P < 0.05 within each time-point.

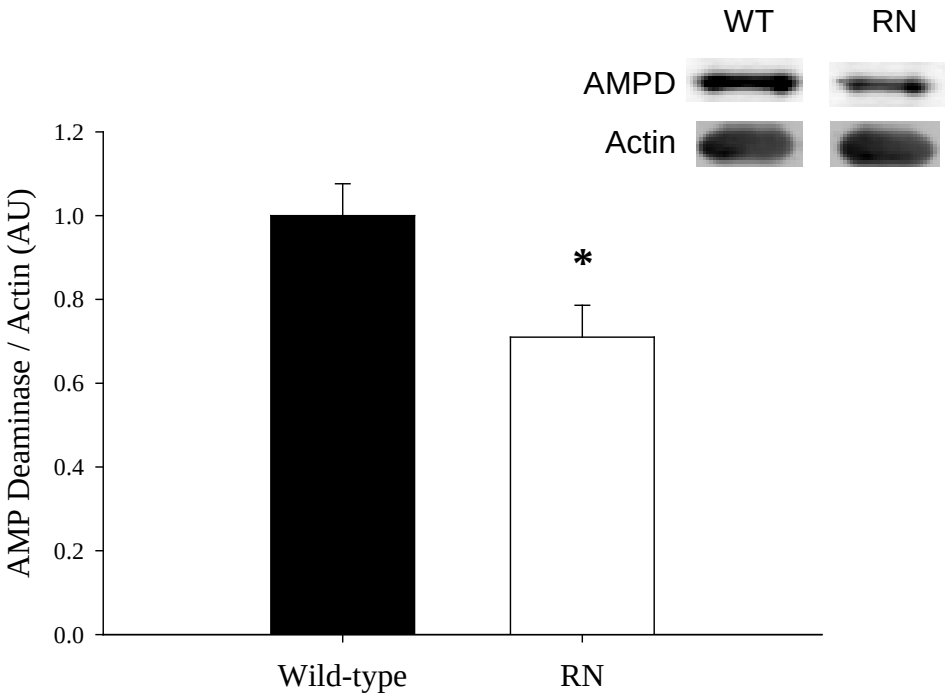

Figure 3-7. Representative image of wild-type and RN- AMP deaminase normalized to actin (upper right). Western blot analysis of AMP deaminase normalized to actin (n = 8 per genotype). Data are mean ± SE. *P < 0.05. RN = AMPK $\gamma 3^{R200Q}$ mutant pigs; WT = Wild-type.

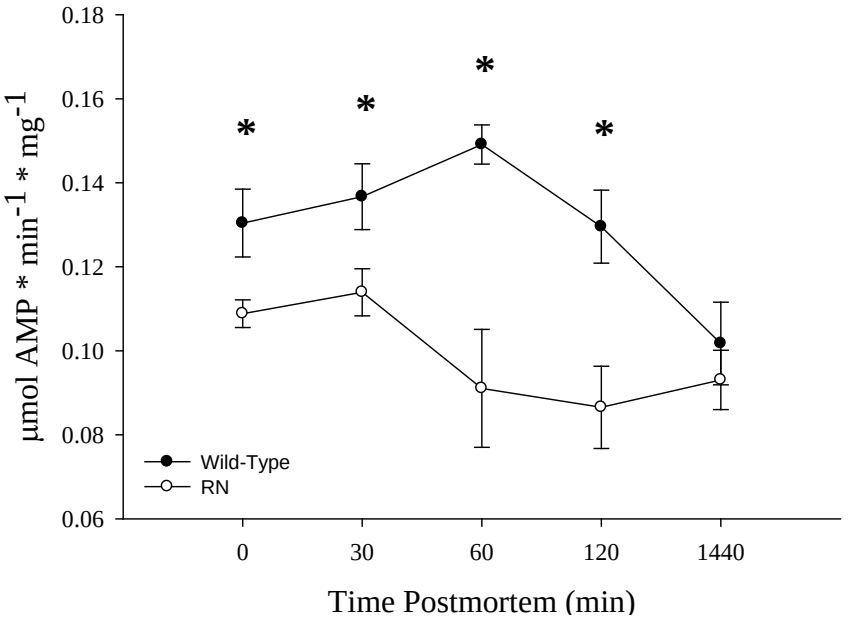

Figure 3-8. Mean postmortem AMP deaminase activity between wild-type and RN^- pigs. Data are mean ± SE. *P < 0.05 within time.

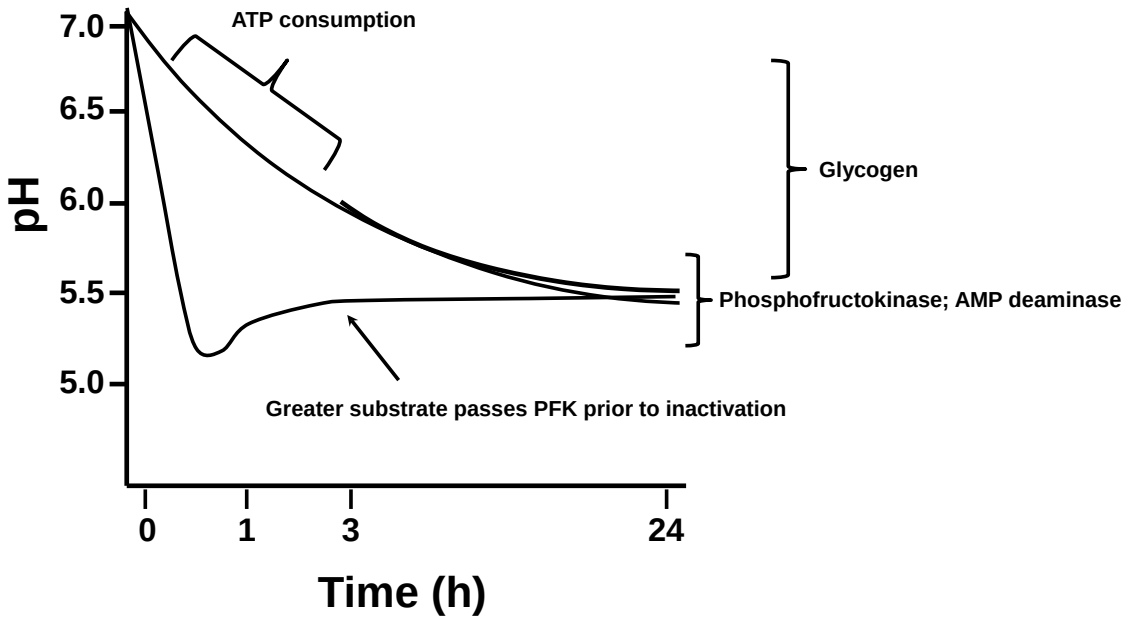

Figure 3-9. Working model of the factors controlling the rate and extent of postmortem metabolism.

Time (min)	0	30	120	240	1440
ATP concentration (mM)					
Control	5.79 ± 0.20	3.66 ± 0.17[a]	0.71 ± 0.20	BD	BD
Pentostatin	5.91 ± 0.19	4.53 ± 0.19[b]	0.57 ± 0.21	BD	BD
ADP concentration (mM)					
Control	0.86 ± 0.04	0.17 ± 0.01[a]	0.11 ± 0.02	0.05 ± 0.01	0.04 ± 0.01
Pentostatin	0.84 ± 0.04	0.30 ± 0.02[b]	0.13 ± 0.04	0.05 ± 0.01	0.04 ± 0.01
AMP concentration (mM)					
Control	0.12 ± 0.01	BD	0.00 ± 0.01[a]	BD	BD
Pentostatin	0.11 ± 0.02	BD	0.03 ± 0.01[b]	BD	BD
IMP concentration (mM)					
Control	0.35 ± 0.03	3.24 ± 0.16[a]	6.95 ± 0.27	6.93 ± 0.99	7.61 ± 0.07
Pentostatin	0.35 ± 0.03	2.09 ± 0.10[b]	7.13 ± 0.24	6.92 ± 0.99	7.46 ± 0.08

Table 3-1. Mean adenonucleotides and inosine monophosphate (mM) of longissimus muscle homogenate. Data are mean ± SE. Means lacking a common superscript differ within a time-point ($P < 0.05$). BD = Below limit of detection.

Table 3-2. Longissimus muscle metabolites at 180 min postmortem from wild-type and RN- pigs. Data are mean ± SE. Mean values within a metabolite without a common superscript (a, b) differ ($P < 0.05$).

		Wild-Type	RN^-
Metabolite	G6P	6.62 ± 0.78 [a]	11.8 ± 0.68 [b]
	ATP	0.82 ± 0.54	0.68 ± 0.43
	ADP	0.55 ± 0.06	0.50 ± 0.06
	AMP	0.06 ± 0.01 [a]	0.12 ± 0.02 [b]
	IMP	5.87 ± 0.50	5.55 ± 0.48

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Chapter 4 - Excess glycogen does not resolve high ultimate pH of oxidative muscle

Abstract

Skeletal muscle glycogen content can impact the extent of postmortem pH decline. Compared to glycolytic muscles, oxidative muscles contain lower glycogen levels antemortem which may contribute to the higher ultimate pH observed at 24 hr postmortem. In an effort to explore further the participation of glycogen in postmortem metabolism, we postulated that increasing the availability of glycogen would drive additional pH decline in oxidative muscles to equivalent pH values similar to the ultimate pH of glycolytic muscles. Glycolysis and pH declines were compared in porcine *longissimus lumborum* (glycolytic) and *masseter* (oxidative) muscles using an *in vitro* system in the presence of excess glycogen. The ultimate pH of the system containing *longissimus lumborum* reached a value similar to that observed in intact muscle. The pH decline of the system containing masseter samples stopped prematurely resulting in a higher ultimate pH which was similar to that of intact *masseter* muscle. To investigate further, we titrated powdered *longissimus lumborum* and *masseter* samples in the reaction buffer. As the percentage of glycolytic sample increased, the ultimate pH decreased. These data show oxidative muscle produces meat with a high ultimate pH regardless of glycogen content and suggest inherent muscle factors associated with glycolytic muscle control the extent of pH decline in pig muscles.

Introduction

Two classical fresh meat quality problems prevail in the meat industry: pale, soft and exudative (PSE) meat and dark, firm and dry (DFD) meat. Dark, firm and dry meat displays

a dark red or purple color with a high ultimate pH. The ultimate pH cutoff for classifying meat as DFD is traditionally thought to be above pH 6.0 (Briskey, 1964), yet some argue as low as 5.87 (Page, Wulf, & Schwotzer, 2001). While this condition is more prevalent in with meat from ruminants, it also occurs in non-ruminants (McVeigh & Tarrant, 1982; Warriss, 1982; Warriss, Bevis, & Ekins, 1989; Warriss, Kestin, Brown, & Wilkins, 1984). Historically, DFD has been attributed to low glycogen content at death (usually due to stress). Stress prior to slaughter often results in a negative linear relationship between ultimate pH and pre-slaughter muscle glycogen, especially when muscle glycogen content is between 0 and 53 μmol/g glycogen in pigs and cattle (Henckel, Karlsson, Jensen, Oksbjerg, & Petersen, 2002; Immonen & Puolanne, 2000). Therefore, muscle glycogen content early postmortem is useful in predicting postmortem pH decline (Warriss, et al., 1989).

Data describing this linear relationship between ultimate pH and glycogen content are collected from studies using the *longissimus lumborum*, after all this muscle is the most economically relevant to consumers interested in fresh meat quality. In pigs, this muscle consists largely of the fast-contracting fibers that are glycolytic in nature. As a result, the ultimate pH of meat produced by this muscle is often near pH 5.5 – 5.6. A strong relationship between glycogen and ultimate pH suggests that glycogen, or more specifically the lack thereof, is responsible for arresting postmortem glycolysis and leading to pork with an elevated ultimate pH. In pigs, especially, a number of muscles produce meat with an ultimate pH at or above pH 5.9, including the adductor, semitendinosus (red portion), gracilis, semimembranosus, gastrocnemius, and masseter (Huff-Lonergan, et al., 2002; Porcine Myology, 2005; Realini, et al., 2013). These muscles, however, contain

lower antemortem glycogen and a higher proportion of slow-contracting fibers than in the *longissimus lumborum*. Therefore, if a linear relationship exists between antemortem muscle glycogen and ultimate pH in all muscles, we hypothesized that providing glycogen in excess will produces meat with an ultimate pH near 5.5 – 5.6. Testing this hypothesis *in vivo* is difficult. Increasing glycogen content of muscle through high starch diets has proven unreliable (reviewed by Fernandez & Tornberg, 1991). Therefore, we tested the aforementioned hypothesis using our *in vitro* system designed to recapitulate postmortem glycolysis (England, Matarneh, Scheffler, Wachet, & Gerrard, 2014; England, Matarneh, Scheffler, Wachet, & Gerrard, 2015).

To conduct this study, a muscle was needed that met two criteria. First, it must be a predominantly oxidative muscle with high ultimate pH. Second, it must be readily available for easy sampling postmortem. The potential options were the *masseter*, the *diaphragm* or red portion of the *semitendinosus*. All three muscles are predominantly slow-contracting and oxidative, but the *masseter* alone exhibits exclusively type I and type IIA fibers (Johnson, White, & Lawrie, 1986; Realini, et al., 2013; Toniolo, et al., 2004; Tuxen & Kirkeby, 1990). While the porcine *masseter* does not contain nearly as high a proportion of type I fibers as cattle (~100%) (Johnson, et al., 1986), it met both criteria for this study. Therefore, the *masseter* was selected over the *diaphragm* or red *semitendinosus*.

Materials and methods

Sample collection

Market-weight pigs (100 – 125 kg) were harvested in the Virginia Tech Meat Center using accepted commercial processing procedures. Following exsanguination, porcine

longissimus lumborum and masseter samples were excised at 5 and 1440 min postmortem. Samples were snap frozen in liquid nitrogen and stored at − 80 °C.

Meat quality characteristics and muscle metabolites

The intact 24 hr samples of the *longissimus lumborum* and *masseter* were analyzed for meat quality and muscle metabolites. Specifically glycogen, glucose 6-phosphate, glucose, and lactate were measured according to Bergmeyer (1984) using a method modified for microplate design (Hammelman, et al., 2003). Muscle pH was measured by powdering the tissue and homogenizing it using a solution containing 150 mM KCl and 5 mM sodium iodoacetate at a 1:8 ratio (wt/vol) (Bendall, 1973). Samples were equilibrated to 25 ^{o}C, centrifuged and measured immediately thereafter using a Thermo Scientific Orion Ross Ultra Semi-Micro glass electrode (Thermo Scientific, Pittsburgh, PA). Both the *longissimus lumborum* and *masseter* were measured for objective color (L*, a*, and b*) using a Minolta colorimeter CR-300 (Konica Minolta Inc.; Osaka, Japan). The mean value of three separate measurements was used for comparisons between muscles.

In vitro buffer system

The 5 min porcine *longissimus lumborum* and *masseter* muscles (n = 6 for each muscle) were powdered in liquid nitrogen and homogenized at a 100 mg / mL (1:10 ratio) into an anaerobic glycolysis buffer containing 10 mM Na_2HPO_4, 5 mM $MgCl_2$, 60 mM KCl, 5 mM ATP, 0.5 mM ADP, 0.5 mM NAD+, 30 mM glycogen, 25 mM carnosine, 30 mM creatine and 10 mM sodium acetate (pH 7.4) (England, et al., 2014; England, et al., 2015). Reaction vessels were stored at 25 ^{o}C for the duration of the trial. Aliquots were removed

from the muscle and reaction buffer homogenate at 0, 60, 120, 240 and 1440 min for further analysis. In a separate study, the ratio of total muscle mass to buffer volume was maintained, but the percent composition of *masseter* and *longissimus lumborum* were varied in order to titrate ultimate pH of the system. In another study, the extent of pH decline for the *masseter* was compared with and without a cocktail of mitochondria electron transport chain inhibitors (2 μM rotenone (Complex I), 1 mM potassium cyanide (Complex IV), and 2 μM oligomycin (Complex V)).

Metabolite analysis

Aliquots were removed from the homogenate for glycogen analysis and mixed with an equal volume of 2.5 M HCl, heated at 90 ^{o}C for 2 h, centrifuged, and the resulting supernatant was neutralized with 1.25 M KOH (Bergmeyer, 1984). Samples were removed from the homogenate for lactate, glucose 6-phosphate, ATP analysis and treated with an equal volume of ice-cold 1 M perchloric acid, centrifuged, and the resulting supernatant was neutralized with 2 M KOH (Bergmeyer, 1984).

Glycogen, lactate, glucose, and glucose 6-phosphate were measured from muscle homogenates using previous methods (Bergmeyer, 1984) modified for microplate volumes (Hammelman, et al., 2003). All reactions occurred in 5 mL borosilicate glass tubes and were analyzed spectrophotometrically at 340 nm in triplicate using 96-well microplates. ATP was separated with an HP Agilent 1100 (Agilent Technologies, Santa Clara, CA) using an Accucore C18 2.6 μm 50 mm x 4.6 mm column (Thermo Scientific, Pittsburgh, PA), detected at 254 nm with gradient separation (Bernocchi, et al., 1994; Williams, Vidt,

& Rinehart, 2008) and quantified using commercially available ATP (Sigma-Aldrich, St. Louis, MO) as a standard.

pH Analysis

Measurement of muscle pH was conducted according to (Bendall, 1973). The muscle homogenate pH was measured similarly with a slight modification. Four volumes of homogenate were transferred to a new tube and one volume of 25 mM sodium iodoacetate and 750 mM KCl (pH 7.0) was added. Samples were equilibrated to 25 ^{o}C, centrifuged and measured immediately thereafter using a Thermo Scientific Orion Ross Ultra Semi-Micro glass electrode (Thermo Scientific, Pittsburgh, PA).

Acid phosphatase activity assay

Acid phosphatase activity was measure according to a modified procedure by Bergmeyer (1974). Briefly, muscle samples were homogenized in 100 mM K_2HPO_4 (pH 7.4) and centrifuged. Aliquots of the supernatant were added to the reaction buffer containing 41 mM sodium citrate (pH 4.8) and 6.9 mM p-nitrophenylphosphate heated to 37 ^{o}C. Following a 10 min incubation, 4 volumes of 100 mM NaOH were added to stop the reaction. The activity was measured in triplicate using 96-well microplates spectrophotometrically at 410 nm and reported as units of activity defined as 1.0 μmole of p-nitrophenylphosphate per minute per g whole tissue at pH 4.8 and 37°C.

Gel electrophoresis and immunoblotting

Muscle tissue was powdered in liquid nitrogen and the proteins solubilized according to Warren, Krzesinski, & Greaser (2003). Solubilized proteins from the *longissimus lumborum* and *masseter* (n = 6 for each muscle) were separated by SDS-PAGE (7%, 10% or 14% acrylamide), transferred to nitrocellulose membranes and immunoblotted with primary antibodies specific for phosphofructokinase, myoglobin, citrate synthase (Abcam, Cambridge, MA), lactate dehydrogenase A, glyceraldehyde 3-phosphate dehydrogenase (Novus Biologicals, Littleton, CO), myosin heavy chain I, and actin (Developmental Studies Hybridoma Bank, Iowa City, IA). Bands were visualized with IRDye fluorescent secondary antibodies (LI-COR Biosciences, Lincoln, NE) and quantified using LI-COR Biosciences Odyssey imaging system and software.

Statistical Analysis

Data are presented as means ± SE. All data were analyzed with a mixed model in JMP (SAS Institute Inc., Cary, NC). Individual animals were recognized as an experimental unit and time-course data were analyzed with a split-plot design. All differences are presented within time. The least squares means were evaluated using a Student's t-test and considered significant at $P \leq 0.05$.

Results and discussion

Meat quality and muscle fiber-type

Meat quality profiling was compared between the intact *longissimus lumborum* and *masseter* (Table 4-1). At 24 hr, the *masseter* exhibited lower lactate formation, lower lightness (L*) value, higher redness (a*) and a greater ultimate pH. These data are

consistent with previous characterization (Realini, et al., 2013). In addition, the abundance of specific metabolic and contractile proteins was measured (Figure 4-1). The *masseter* contained increased myosin heavy chain type I isoform (slow-contracting), lower glycolytic enzyme abundance (lactate dehydrogenase, phosphofructokinase-1, and glycerol 3-phosphate dehydrogenase; $P < 0.0001$), greater mitochondrial enzyme capacity (citrate synthase; $P < 0.0001$) and greater myoglobin ($P < 0.0001$). All proteins analyzed further suggested the *masseter* is a red muscle that contains a high proportion of slow-contracting muscle fibers that possess a more oxidative metabolic profile than the *longissimus lumborum*.

In vitro buffer system

Using this system, we compared the extent of glycolysis and pH decline between the *longissimus lumborum* and *masseter*. Glycogen was included at 30 mM and decreased ($P < 0.0001$) with time in both treatments. However, no treatment differences ($P = 0.8067$) were found at any time-point (Figure 4-2). Furthermore, glycogen content was not depleted in either treatment at 1440 min which indicated that glycogen was not limiting.

The extent of pH decline differed between the muscles. The *masseter* pH was higher at 0 min and the difference was maintained through the entire period (Figure 4-3A; $P \leq 0.0145$). The reaction buffer was initially adjusted to pH 7.4. When added, the *longissimus lumborum* decreased the initial pH to 6.8. However, the *masseter* only decreased the pH to 7.0 either because the tissue had a higher initial pH or a reduced amount of buffering compounds with a pKa below 7.2 compared to the *longissimus lumborum*. Regardless, when initial pH was normalized to 0 min, net pH decline still maintained the

difference with time (Figure 4-3B). In a previous study, we concluded that phosphofructokinase-1 activity ultimately terminates postmortem glycolysis and pH decline between pH 5.9 – 5.5 (England, et al., 2014). The ultimate pH of the *masseter* treatment was 5.92 ± 0.09. In comparison to the 24 hr intact *masseter* (Table 1), there is a greater pH decline in vitro. Yet, glycogen was not depleted *in vivo* and indicates postmortem glycolytic flux could have continued. This premature termination of pH decline *in vivo* was likely due to the cold temperatures used in pork processing. Lactate formation followed pH decline. Both total and net lactate was greater in the *longissimus lumborum* than the *masseter* ($P \leq 0.05$; Figure 4-4). Lactate also correlated strongly with pH across the entire reaction period for the *longissimus lumborum* ($R^2 = 0.71$; $P < 0.0001$) and *masseter* ($R^2 = 0.79$; $P < 0.0001$). Combined, these data show that pH and glycolysis is arrested in the *masseter* and stops at a higher ultimate pH even in the presence of excess glycogen.

One surprising finding was the discrepancy between glycogen disappearance and lactate formation. Lactate formation was dramatically greater in the *longissimus lumborum*, but glycogen catabolism was not different. Therefore, we investigated glucose 6-phosphate and glucose content (Figure 4-5). In the *longissimus lumborum*, glucose 6-phosphate was greater ($P < 0.0001$) at 120, 240 and 1440 min. However, glucose was greater ($P < 0.001$) in the *masseter* at 240 and 1440 min. To explain this phenomenon, we postulated that glucose was being produced in a manner not normally present during *in vivo* postmortem metabolism. Normally, glycogen debranching enzyme (EC 2.4.1.25; EC 3.2.1.33) releases glucose molecules that are not phosphorylated by hexokinase postmortem and thus glucose accumulates with time postmortem. Alternatively, an additional enzyme may be producing

glucose in our system. The two likely enzymes responsible were glucose 6-phosphatase (EC 3.1.3.9) or acid phosphatase (EC 3.1.3.2). Glucose 6-phosphatase is a gluconeogenic enzyme that catalyzes the conversion of glucose 6-phosphate to glucose and is found in liver and kidney tissue, but has little significant activity (if any) in muscle tissue (van Schaftingen & Gerin, 2002). Alternatively, acid phosphatase is a lysosomal enzyme that functions to liberate inorganic phosphate from orthophosphoric monoesters like glucose 6-phosphate as pH declines. Indeed, the *masseter* contained greater ($P < 0.001$) acid phosphatase enzymatic activity (Figure 4-6). Others showed that acid phosphatase is more abundant in oxidative muscles of guinea pigs and mice (Peter, Kar, Barnard, Pearson, & Edgerton, 1972; Vihko, Salminen, & Rantamaki, 1979) and these data further support the oxidative status of the *masseter* and suggest that if lysosomes are disrupted postmortem, the conversion of glucose 6-phosphate to glucose may occur, altering the notion that glucose formation is exclusively caused by glycogen catabolism. However because other lysosomal-based enzymes like cathepsin play little role in postmortem proteolysis (Ouali, 1992), it suggests that the increased activity in our system is an *in vitro* manifestation only and acid phosphatase likely plays little role *in vivo*.

The *masseter* had lower ($P = 0.0305$) ATP at 0 min but greater ($P = 0.0419$) ATP at 240 min (Figure 4-6). However, the overall ATP hydrolysis was not different ($P = 0.3941$) between the muscles. These results suggest ATP hydrolysis is not substantially different between the muscles. However, due to the increased lactate production by the *longissimus lumborum*, the net overall number of ATP molecules generated by glycolysis was greater. Previous work from our lab indicated that while myofibrillar ATPase activity was significantly greater in a predominantly glycolytic muscle (white *semitendinosus*) than

an oxidative muscle (red *semitendinosus*), overall activity was not appreciably different between glycolytic and oxidative muscles (Bowker, Grant, Swartz, & Gerrard, 2004) at least compared to the difference in glycolytic capacity. Therefore, the rate of ATP hydrolysis between the muscles is more similar than the rate of glycolysis.

When these data are synthesized in totality, the differences between lactate formation, pH decline and ATP disappearance suggest glycolytic capacity (or lack thereof) explains the high ultimate pH of oxidative muscle. During postmortem metabolism, the glycolysis and phosphagen pathways work in conjunction to provide reactants and products for each other. These systems presumably function until a reactant is limiting or pH is lowered to the range where phosphofructokinase activity is inactivated (England, et al., 2014). Because ATP disappearance was similar between to two-muscle types, the rate of glycolytic flux is responsible for converting carbohydrate to glycolytic intermediates prior to inactivation of phosphofructokinase. Glycolytic muscle has a high glycolytic capacity and a faster rate of postmortem glycolysis resulting in an ultimate pH near the bottom of the functional range of phosphofructokinase. The glycolytic capacity of oxidative muscle is low and unable to 'keep up' with the phosphagen system. Even in the presence of excess glycogen, pH decline is terminated near the upper end of the pH range near pH 5.9. Thus, the ultimate pH of meat in the presence of excess glycogen is controlled by the glycolytic capacity of the muscle that is determined prior to slaughter.

To follow-up, we tested whether glycolytic capacity linearly controlled ultimate pH. The percent composition of muscle was titrated in the *in vitro* system. We maintained a consistent muscle mass to volume ratio, but varied the percent composition between powdered pre-rigor *longissimus lumborum* and *masseter* and measured ultimate pH. The

ultimate pH of the 0% *longissimus lumborum* (i.e. 100% *masseter*), was 5.95 ± 0.03. The ultimate pH decreased ($P < 0.05$) in a curvilinear fashion until a 50:50 ratio was reached and did not decrease further from that point (Figure 4-8). These data show that small increases in glycolytic enzymatic capacity in a red muscle impact ultimate pH of the muscle. These data may help further the correlation between increased oxidative or fast-oxidative fibers and elevated ultimate pH (Chang, et al., 2003; Gil, et al., 2003; Kang, et al., 2011; Maltin, et al., 1997; Ryu, et al., 2008). In these studies, animals with increased oxidative (type I) or fast-oxidative fibers (type IIA) produced meat with an elevated ultimate pH. Because the muscle studied was the *longissimus lumborum*, the elevated ultimate pH improved fresh meat quality. Therefore, better pork quality can be produced by selecting for an increase in the percentage of oxidative fibers in the *longissimus lumborum*.

These findings may extend into fresh beef quality. As stated earlier, DFD meat tends to be a larger problem in ruminants than non-ruminants. Therefore, when meat exhibits an ultimate pH near 6.0, it is classified as DFD and downgraded. This is usually attributed to low glycogen antemortem either through poor nutrition or stress. However, it may be possible that muscle-fiber type plays as much of a roll as glycogen content. Beef muscles are redder and more oxidative than porcine muscles. Therefore, transitioning away from glycolytic capacity may explain as much for the variation in ultimate muscle pH as measuring glycogen content. This may be especially true for grain versus grass fed animals. The *longissimus lumborum* from extensive feeding systems (i.e. roughage and pasture diets) had a higher ultimate pH (Vestergaard, Oksbjerg, & Henckel, 2000). In addition, *longissimus lumborum* from grass-fed animals exhibit increased myosin heavy chain type

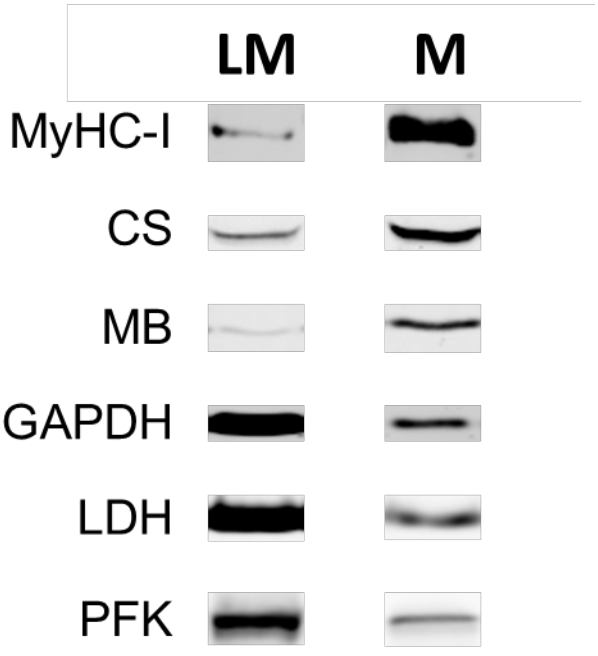
IIA and citrate synthase activity with decreased fast myosin heavy chain isoform and muscle glycogen and therefore a decreased capacity for glycolytic flux. Furthermore, grass-fed beef is darker in color due to higher myoglobin concentration and exhibits a slower rate of postmortem pH decline (Bidner, et al., 1986; Bidner, Schupp, Montgomery, & Carpenter, 1981; French, et al., 2001; Reagan, Carpenter, Bauer, & Lowrey, 1977) which both are indicators of a more oxidative phenotype. All these findings are consistent with the *in vitro* work from this study. Therefore, muscle fiber-type and metabolic capacity may explain quality variation between grass-fed and grain fed animals.

These results must also be addressed in light of the AMPK $\gamma3^{R200Q}$ (RN^-) mutant pigs. Muscle from these mutant pigs exhibits a slower contractile speed (Park, Gunawan, Scheffler, Grant, & Gerrard, 2009) and possesses lower lactate dehydrogenase activity with greater citrate synthase and 3-hydroxyacyl-CoA dehydrogenase activity in the *longissimus lumborum* (England, et al., 2015; Lebret, et al., 1999; Scheffler, et al., 2014). In addition, these mutant pigs have 50% greater muscle mitochondria (Scheffler, et al., 2014), epitomizing an oxidative type of muscle. Based on this study, therefore, mutant pigs should produce meat with a higher ultimate pH than their wild-type counterparts or at least not different. However, these animals consistently produce meat with an abnormally low ultimate pH of 5.2 - 5.3 that is often classified as acid meat (Milan, et al., 1996). The low ultimate pH is produced from a net increase in glycolytic flux (unpublished data). Therefore, an overriding mechanism must be able to extend pH decline, postmortem glycolysis or both. To date, the exact mechanism is unknown. While these animals deposit 70% more glycogen in the *longissimus lumborum* than wild-type pigs (Milan, et al., 2000), we have shown the muscle also contains greater glycogen phosphorylase and lower AMP

deaminase activity (England, et al., 2015; Granlund, Jensen-Waern, & Essén-Gustavsson, 2011). Both enzymes have been implicated in extending postmortem pH decline (Scopes, 1974). Alternatively, additional mitochondria may play a role in postmortem metabolism and extend postmortem pH decline. Our lab has recently reported that mitochondria can extend pH decline and postmortem glycolysis *in vitro* (Scheffler, Matarneh, England, & Gerrard, In Review). This is an exciting finding because RN^- muscle contains greater number of mitochondria and the mitochondria have greater maximal, ADP-stimulated oxygen consumption rate compared to wild-type animals (Scheffler, et al., 2014). To address this issue, we investigated the role the mitochondria play in the *in vitro* glycolysis of the *masseter*. We included a cocktail of mitochondrial inhibitors in our *in vitro* glycolytic system with the *masseter* muscle. As evident by the difference in citrate synthase abundance (Figure 4-1), the *masseter* contains substantially more mitochondria. This study was conducted with frozen *masseter* muscle with no added mitochondria. The pH declines of those treatments including mitochondria inhibitors were arrested early at an ultimate pH approximately 0.2 higher than controls (Figure 4-9). Furthermore, the difference in ultimate pH deviated late postmortem which is also consistent with the RN^- pig (Copenhafer, Richert, Schinckel, Grant, & Gerrard, 2006). While not specifically outlining the role the mitochondria play in postmortem metabolism, these data raise the possibility that mitochondria participate in energy production postmortem and play a role in dictating fresh meat quality and controlling ultimate pH.

Conclusions

These data indicate that in the presence of excess glycogen, oxidative muscle still produces high ultimate pH meat. Prior to this study, high ultimate pH meat in oxidative muscles has been suggested to be directly caused by a lack of antemortem glycogen levels. Rather, both the *in vivo* and *in vitro* studies suggest porcine oxidative muscles can and do stop postmortem pH decline in the presence of excess glycogen. Instead, the glycolytic capacity of the muscle dictates ultimate pH and subsequently quality. To that end, we have updated the working model (England, et al., 2014; England, et al., 2015) that outlines the factors that control both the rate and extent of postmortem pH decline (Figure 4-10).



A

B

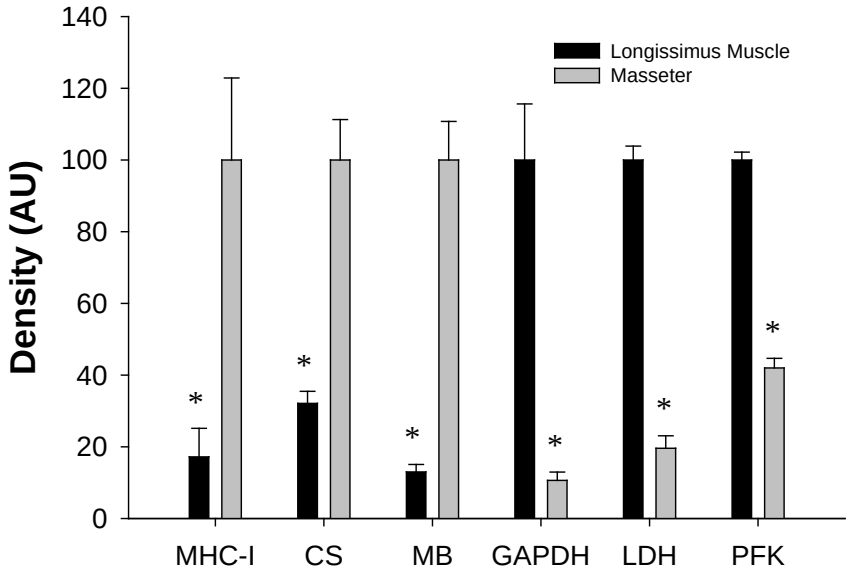


Figure 4-1. Representative western blot of protein abundance (A) of *longissimus lumborum* (LM) and *masseter* (M) muscles (n = 6 per muscle). MyHC-1: Myosin heavy chain type I isoform. CS: Citrate synthase. MB: Myoglobin. GAPDH: Glycerol 3-phosphate dehydrogenase. LDH: Lactate dehydrogenase. PFK: Phosphofructokinase-1. Relative immunoblot band density (B) between muscles. Data are mean ± SE. **P* < 0.0001.

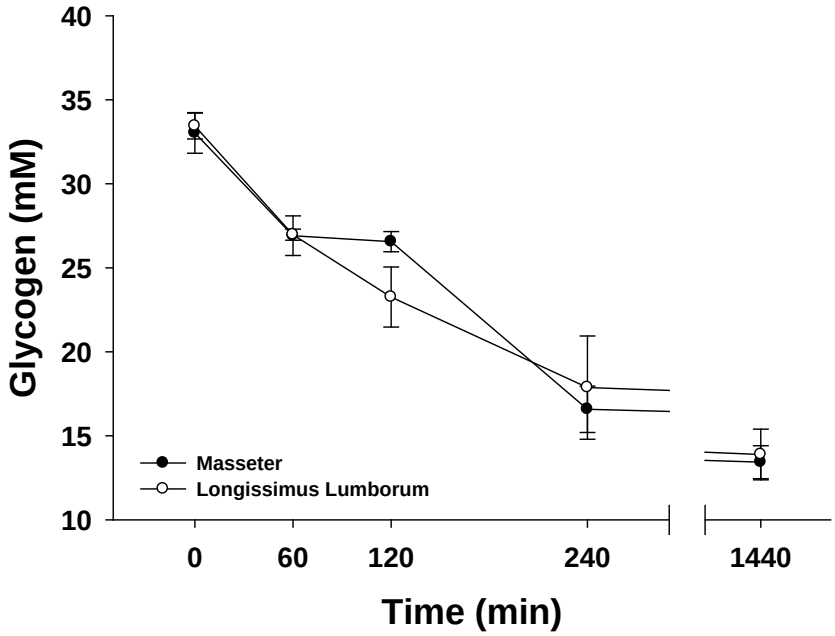


Figure 4-2. Mean glycogen (mM) of the reaction buffer and longissimus lumborum or masseter (n = 6 per muscle). Data are mean ± SE. *P < 0.05 within time.

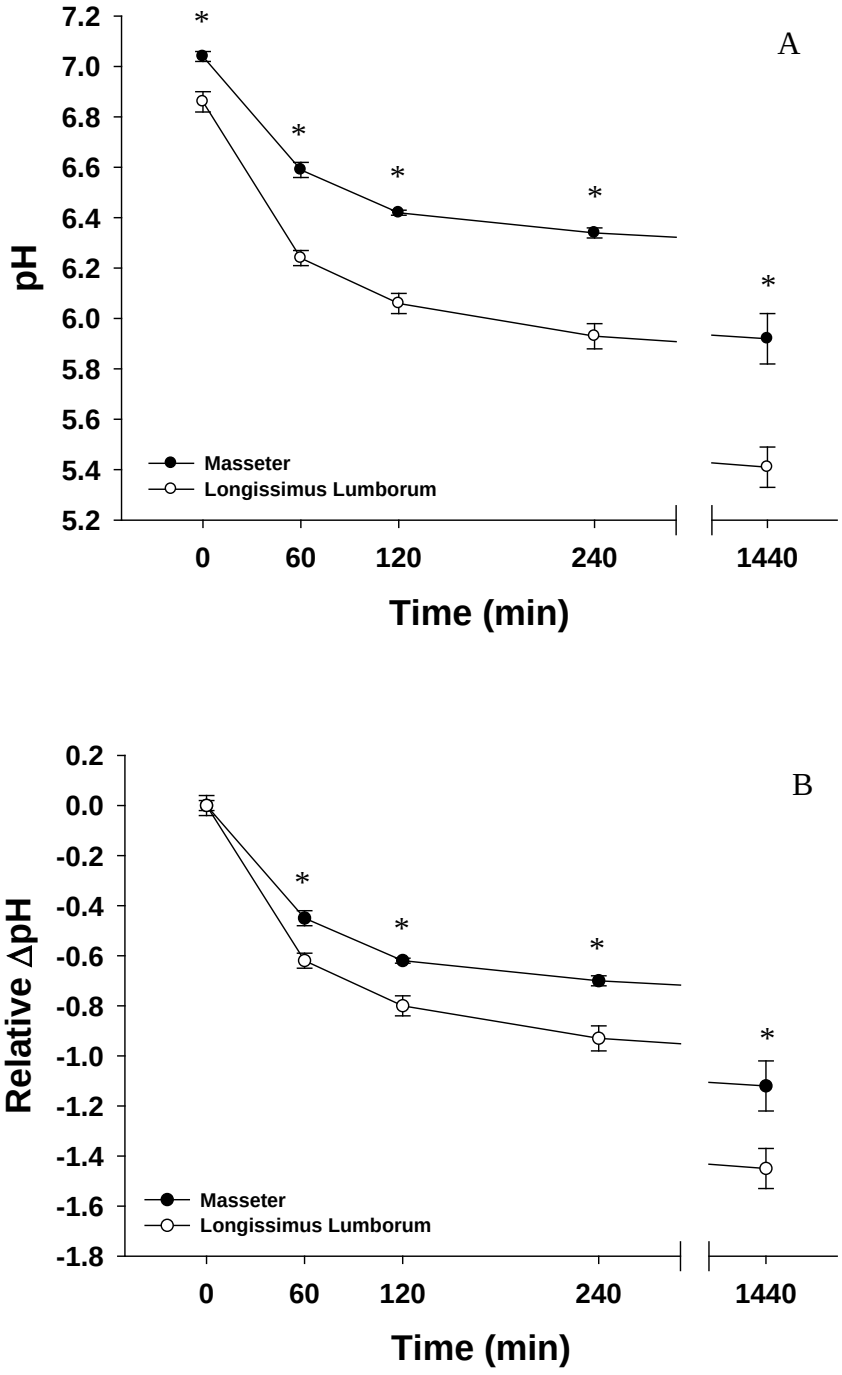


Figure 4-3. Mean pH decline (A) and normalized to 0 min pH decline (B) of the reaction buffer and longissimus lumborum or masseter (n = 6 per muscle). Data are mean ± SE. *P < 0.05 within time.

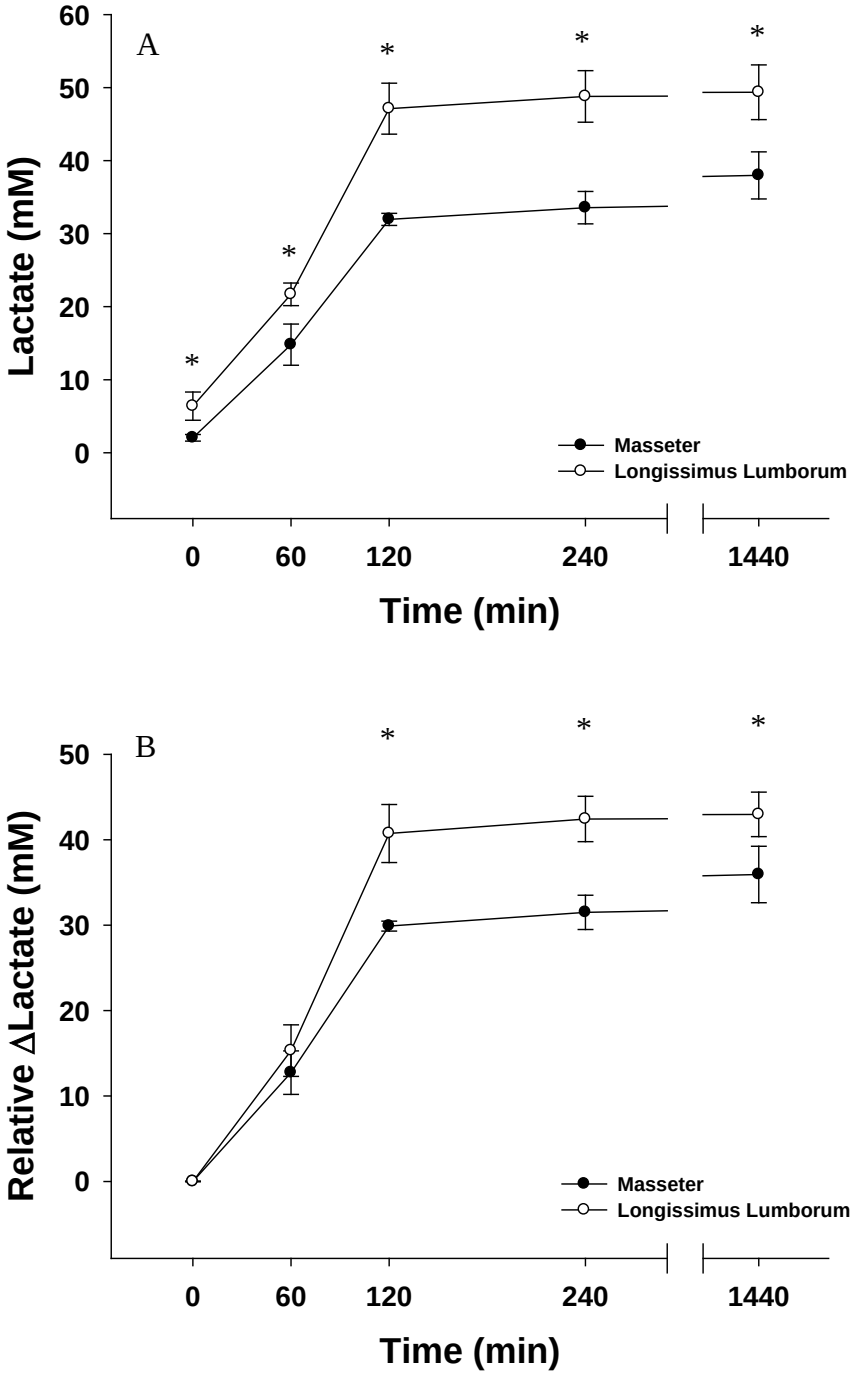


Figure 4-4. Mean lactate (mM; A) and normalized to 0 min lactate (mM; B) of the reaction buffer and longissimus lumborum or masseter (n = 6 per muscle). Data are mean ± SE. *P < 0.05 within time.

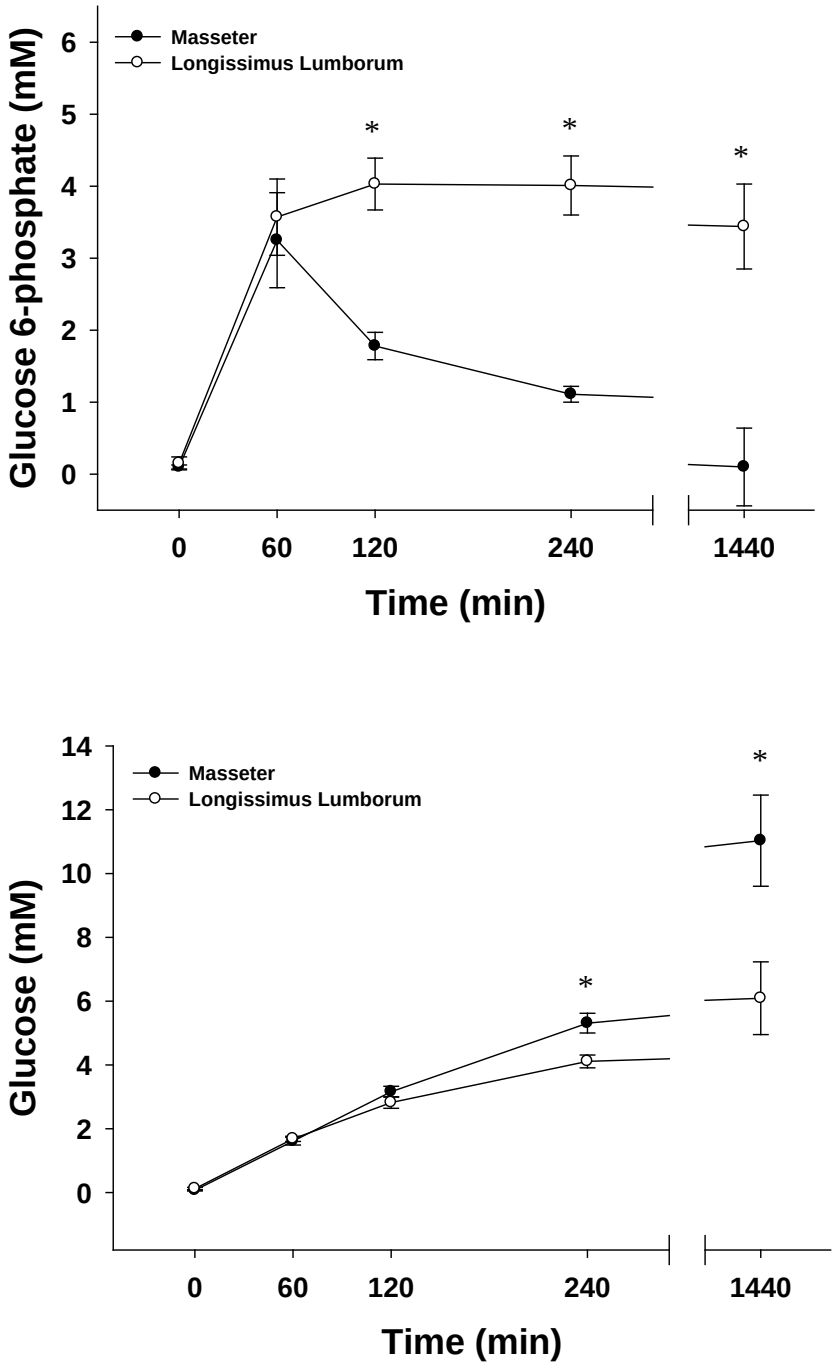


Figure 4-5. Mean glucose 6-phosphate (A; mM) and glucose (B; mM) of the reaction buffer and longissimus lumborum or masseter (n = 6 per muscle). Data are mean ± SE. *P < 0.05 within time.

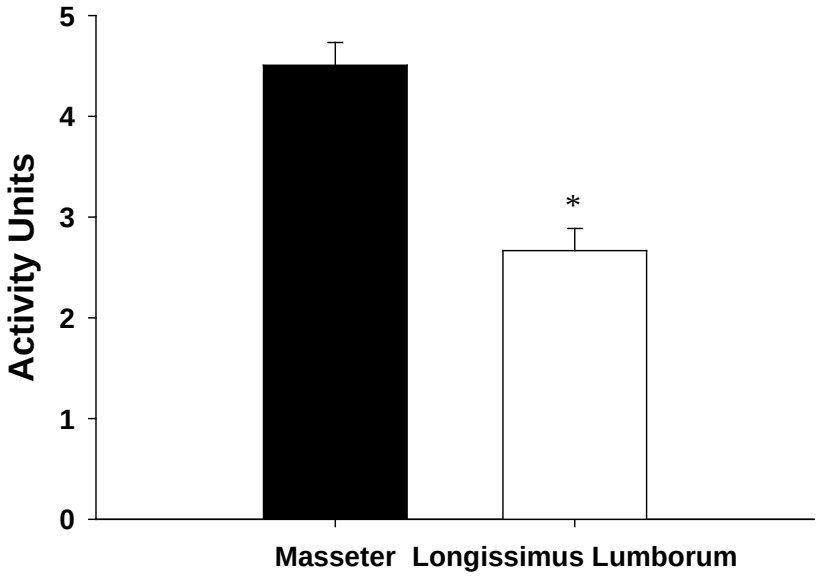


Figure 4-6. Mean acid phosphatase activity of the longissimus lumborum or masseter (n = 8 per muscle). One unit of activity is defined as 1.0 μmole of p-nitrophenylphosphate per minute per g whole tissue at pH 4.8 and 37°C. *P < 0.05

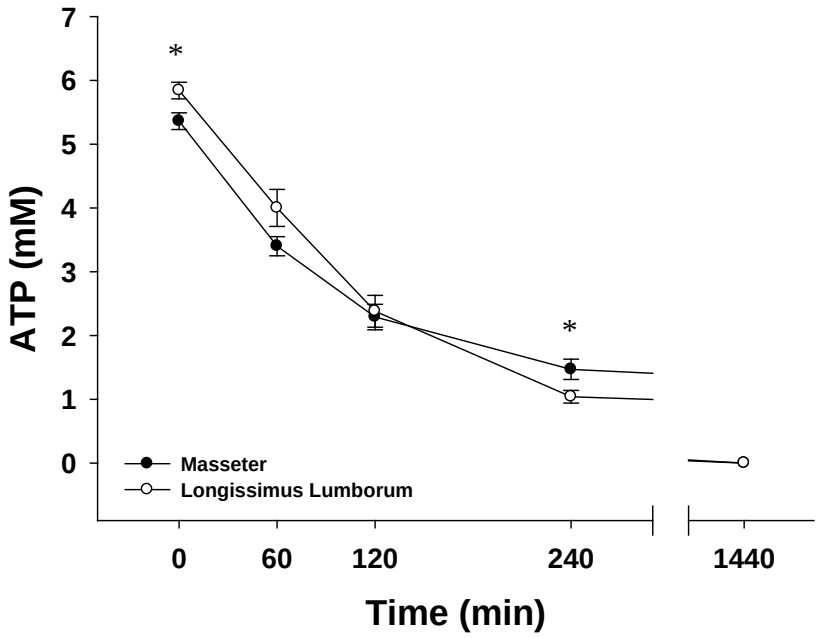


Figure 4-7. Mean ATP (mM) of the reaction buffer and longissimus lumborum or masseter (n = 6 per muscle). Data are mean ± SE. *P < 0.05 within time.

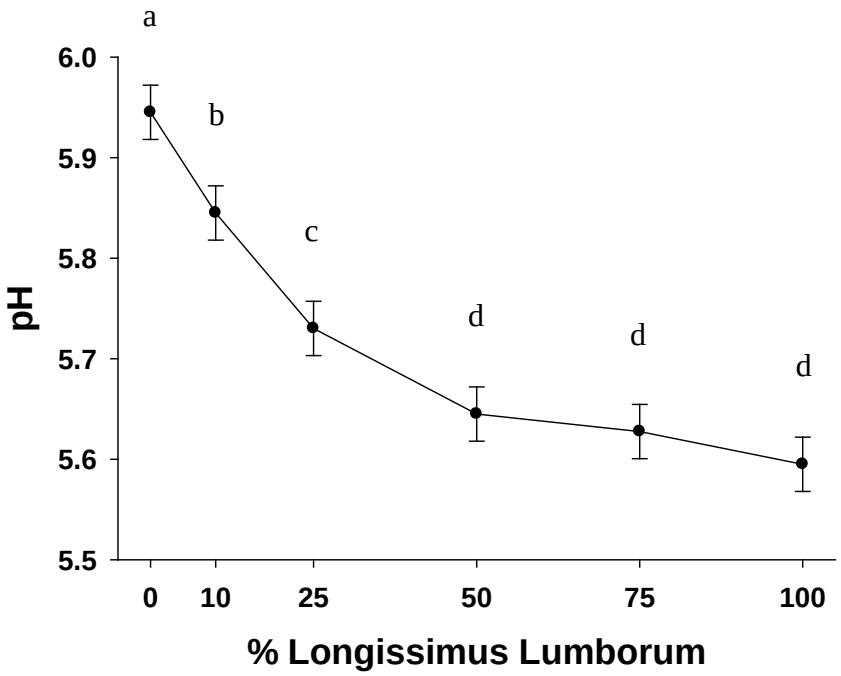


Figure 4-8. Mean ultimate pH of the longissimus lumborum and masseter mixtures and reaction buffer at specified ratios (n = 4 per muscle). Data are mean ± SE. Mean values without a common superscript (a,b,c,d) are different ($P < 0.05$).

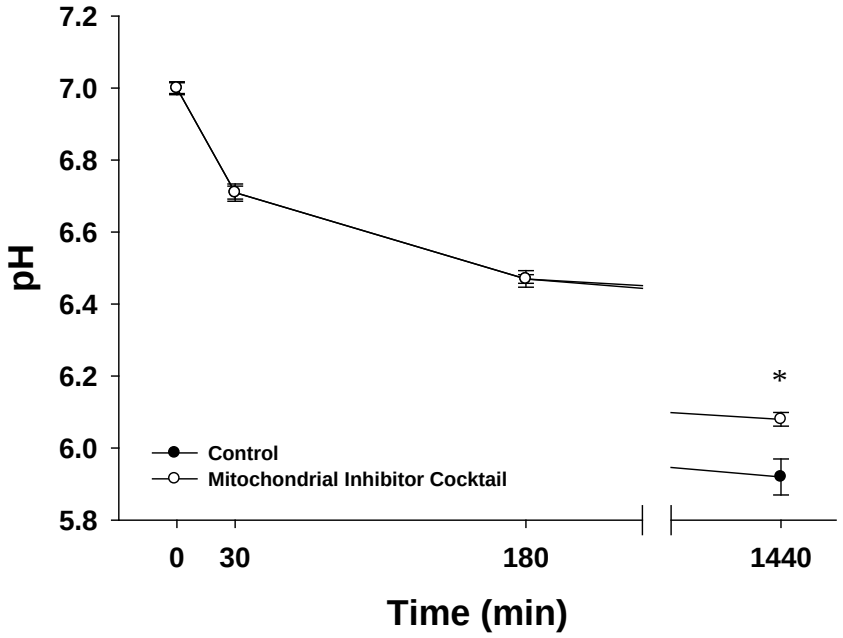


Figure 4-9. Mean of the reaction buffer and masseter mixture with and without the mitochondrial electron transport chain inhibitor cocktail (n = 6 per treatment; 2 μM rotenone, 1 mM potassium cyanide, and 2 μM oligomycin). Data are mean ± SE. *P < 0.05 within time

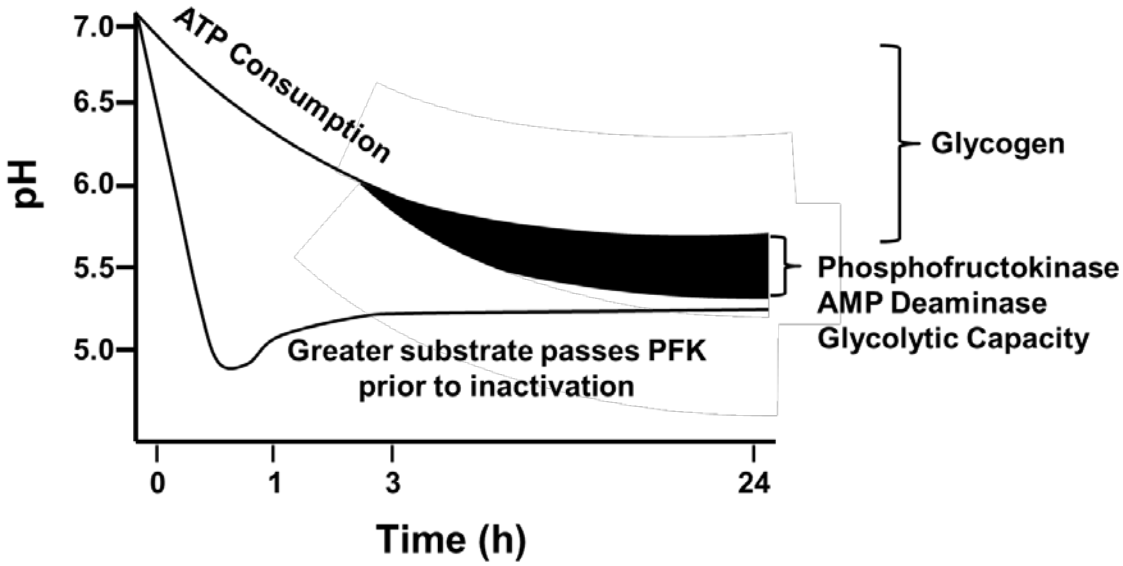


Figure 4-10. Working model of the factors controlling the rate and extent of postmortem metabolism.

Table 4-1. Meat quality characteristics of the *longissimus lumborum* and *masseter* (n = 5 per muscle) at 24 hr postmortem. All measurements are from 24 hr samples. Data are mean ± SE. Mean values without a common superscript (a,b) within a row are different ($P < 0.05$).

	Longissimus Lumborum	Masseter
pH_{24}	5.47 ± 0.01^{a}	6.12 ± 0.04^{b}
$Glycogen_{24}$	16.41 ± 1.98	10.37 ± 3.53
$G6P_{24}$	6.76 ± 1.37	5.42 ± 1.15
$Glucose_{24}$	7.06 ± 0.66^{a}	2.89 ± 0.41^{b}
$Lactate_{24}$	94.14 ± 1.77^{a}	46.77 ± 3.58^{b}
L*	64.14 ± 1.60^{a}	37.89 ± 0.65^{b}
a*	11.14 ± 0.73^{a}	25.48 ± 0.95^{b}
b*	7.06 ± 0.62	6.74 ± 0.24

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Chapter 5 – Chronic activation of AMP-activated protein kinase increases MCT2 and MCT4 expression in skeletal muscle of pigs

Abstract

To date, 14 members of the solute carrier 16 (SLC16) gene family have been identified (Halestrap, 2013). These genes encode for monocarboxylate transporters (MCT). MCTs play an important role in metabolism because they are proton-linked transporters of monocarboxylate molecules like lactate, pyruvate, and ketone bodies across plasma membranes (Poole & Halestrap, 1993). MCT isoform distribution varies considerably between organism and tissue types (Bonen, 2000; Halestrap & Meredith, 2004). In skeletal muscle, the predominant isoforms expressed are MCT1 and MCT4 and to a lesser extent MCT2 (Halestrap & Meredith, 2004). MCT1 is expressed in most muscle of most species, but in skeletal muscle, it is generally more abundant in red, oxidative muscles (Bonen, 2000). MCT2 is expressed in muscle, but varies considerably between species (Halestrap, 2013). MCT2 has only been detected in skeletal muscle from hamsters, pigs, and rats (Benton, Campbell, Tonouchi, Hatta, & Bonen, 2004; Jackson, Price, Carpenter, & Halestrap, 1997; Parkunan, et al., 2015; Sepponen, Koho, Puolanne, Ruusunen, & Pösö, 2003). MCT1 and MCT2 function to transport lactate or ketone bodies into a cell for oxidation (i.e. heart and skeletal muscle) or gluconeogenesis (i.e. liver and kidney) (Halestrap, 2012). MCT4 increases in fast, glycolytic muscles as it primarily functions to transport lactate out of the cell (Bonen, 2000; Dimmer, Friedrich, Lang, Deitmer, & Broer, 2000; Fox, Meredith, & Halestrap, 2000; Pilegaard, Terzis, Halestrap, & Juel, 1999). However, all transporters export lactate out of the cell under hypoxic conditions (Halestrap,

2012). When lactate is produced in a white, glycolytic fiber, it is exported out by MCT4 and imported by MCT1/MCT2 into a red, oxidative fiber for mitochondrial respiration (Halestrap & Meredith, 2004).

MCT isoform expression varies will metabolic demands in muscle (Halestrap, 2011). While results vary with duration and intensity, exercising typically increases MCT1 and(or) MCT4 expression in skeletal muscle (Coles, Litt, Hatta, & Bonen, 2004; Dubouchaud, Butterfield, Wolfel, Bergman, & Brooks, 2000; H. Pilegaard, et al., 1999). Acute bouts of exercise increase MCT expression by activating AMP-activated protein kinase (AMPK). AMPK exists as a heterotrimeric complex comprised of a catalytic α-subunit and regulatory β- and γ-subunits and functions as a sensor to monitor energy charge within the muscle (Hardie & Sakamoto, 2006). An increased AMP:ATP ratio is 'sensed' by AMPK to increase energy producing pathways and down-regulate energy consuming pathways (Hardie, Ross, & Hawley, 2012). When activated, AMPK directly phosphorylates peroxisome-proliferator-activated receptor γ coactivator 1 (PGC-1α) which regulates gene expression to increase mitochondrial biogenesis and oxidative capacity (Jäger, Handschin, St.-Pierre, & Spiegelman, 2007; Zong, et al., 2002). PGC-1α regulates MCT1 expression (Benton, et al., 2008; Hashimoto, Hussien, Oommen, Gohil, & Brooks, 2007). While the connection between AMPK and MCT4 is less established, a number of regulatory mechanisms have been proposed. Acute AMPK activation by exercise or with the pharmacological agent 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR) increased MCT1 and(or) MCT4 expression in the muscle of humans and rats (Furugen, et al., 2011; Hamada & Takimoto, 2013; Thomas, Bishop, Lambert, Mercier, & Brooks, 2012). To our knowledge, the relationship between AMPK and MCT2 expression

has never been tested, likely due to the limited number of species expressing MCT2 in skeletal muscle. It is unclear how chronic activation of AMPK alters the expression of MCTs in skeletal muscle.

Chronic activation of AMPK occurs in pigs possessing the AMPK $\gamma3^{R200Q}$ mutation. This mutation arises from an arginine to glutamine substitution in the muscle specific γ3 regulatory subunit of the protein (Milan, et al., 2000). This skeletal muscle specific subunit is highly expressed in white, glycolytic muscle like the longissimus lumborum. The resulting mutation is a gain in function mutation and alters the physiology of the muscle resulting in a simultaneous increase in glycolytic and oxidative capacity (England, Matarneh, Scheffler, Wachet, & Gerrard, 2014; Granlund, Jensen-Waern, & Essén-Gustavsson, 2011; Lebret, et al., 1999; Park, Gunawan, Scheffler, Grant, & Gerrard, 2009; Scheffler, et al., 2014). Similar gain-in-function mutations have been characterized in AMPK $\gamma3^{R2225Q}$ transgenic mice and AMPK $\gamma3^{R2225W}$ humans (Barnes, et al., 2005; Barnes, et al., 2004; Costford, et al., 2007; Garcia-Roves, Osler, Holmström, & Zierath, 2008). Therefore, using AMPK $\gamma3^{R200Q}$ pigs, we intended to determine the impact of chronic activation of AMPK on MCT1, MCT2 and MCT4 in skeletal muscle.

Materials and methods

Genotyping

Pigs were genotyped for the AMPK $\gamma3^{R200Q}$ mutation according to Copenhafer, Richert, Schinckel, Grant, and Gerrard (2006). Briefly, DNA was isolated from tissue and used for PCR amplification. The primers (5'-3') were AAATGTGCAGACAAGGATCTC (forward) and CCCACGAAGCTCTGCTT (reverse). PCR products were digested with

*Bsr*BI at 37ºC overnight and separated using a 2% agarose gel. The AMPK $\gamma3^{R200Q}$ mutation is dominant. Therefore, both homozygous mutants (RN^-/RN^-) and heterozygous (RN^-/rn^+) animals were used.

Animals

All animals were bred and reared at the Virginia Tech Swine Center in accordance with the Institutional Animal Use and Care Committee. After reaching compositional maturity (100 – 125kg), animals were transported to the Virginia Tech Meat Science Center and euthanized. Immediately following exsanguination, samples (~20 g) from the longissimus lumborum, masseter, heart, spleen, liver, lung, kidney, and small intestines were excised and frozen in liquid nitrogen and stored at -80 ºC for qPCR or whole muscle protein electrophoresis. Excised muscle was also left unfrozen and used for mitochondrial extraction. These muscles were chosen based on rapid sampling postmortem, oxidative or glycolytic capacity of the muscles, and based on the muscles investigated a previous report concerning MCT distribution in skeletal muscle (Sepponen, et al., 2003).

Relative transcript abundance using real-time PCR

Total RNA was isolated from the longissimus lumborum and masseter using the Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA). RNA concentration was quantified with a Nanodrop Spectrophotometer. RNA was reverse-transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Grand Island, NY). Reactions (20 μL) were performed in triplicate in 96-well microplates using the 7500 Fast Real Time PCR System (Life Technologies, Grand Island, NY). The PCR master mix

consisted of 1X Fast SYBR Green Master Mix (Life Technologies, Grand Island, NY), 1000 nM of each primer, and 200 ng of cDNA. Primers were designed using Primer-BLAST (National Center for Biotechnology Information) for MCT1, MCT2, MCT4 (Table 5-1). All MCT genes were normalized to β-actin. Relative transcript abundance was determined by the comparative $\Delta\Delta C_T$ method.

Gel electrophoresis and immunoblotting

Muscle tissue was powdered in liquid nitrogen and the proteins solubilized according to Warren, Krzesinski, and Greaser (2003). Solubilized proteins from the muscles (n = 6 for each muscle and genotype combination) were separated by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with primary antibodies specific for MCT2 (rabbit polyclonal; AB3542; EMD Millipore, Billerica, MA), MCT4 (rabbit polyclonal; ab74109; Abcam, Cambridge, MA), succinate dehydrogenase subunit A (SDH-A; mouse monoclonal; ab14715; Abcam, Cambridge, MA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; mouse monoclonal; NB300-221; Novus Biologicals, Littleton, CO), and glucose transporter type 4 (GLUT4; mouse monoclonal; 2213; Cell Signaling Technology, Inc., Danvers, MA). Blots were visualized with IRDye fluorescent secondary antibodies (LI-COR Biosciences, Lincoln, NE) and quantified using LI-COR Biosciences Odyssey imaging system and software. Reversible ponceau S staining was used as a loading control.

Mitochondrial extraction, electrophoresis and immunoblotting

Mitochondria were isolated from wild-type and AMPK $\gamma3^{R200Q}$ longissimus lumborum and masseter using a modified method by Scheffler, et al. (2014). Briefly, freshly excised muscle samples were added in ice-cold homogenization buffer (5 ml/g fresh muscle; 180 mM KCl, 100 mM sucrose, 50 mM Tris, 10 mM EDTA, 5 mM $MgCl_2$, and 1 mM K-ATP, pH 7.4). Muscle was minced with scissors followed by the addition of a protease (subtilisin A; 5 mg/mL). Muscle mixtures were homogenized with a glass-Teflon homogenizer then filtered with two layers of cheese cloth. The homogenate was centrifuged at 1,000 *g* for 10 min at 4^{o}C followed by a second filtration of the supernatant through two layers of cheesecloth. The filtered supernatant was centrifuged again 8,000 *g* for 10 min at 4^{o}C. The resulting supernatant was discarded and the mitochondrial pellet was suspended in mannitol-sucrose medium (220 mM mannitol, 70 mM sucrose, 10 mM Tris-HCl, and 1 mM EGTA, pH 7.4). Mitochondrial protein content was measured using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Equal amounts of mitochondrial protein were solubilized with the same buffer used in whole muscle electrophoresis and separated with SDS-PAGE using Any kD gels (Bio-Rad, Hercules, CA). Proteins were transferred and subjected to immunoblotting as described in the previous methods section.

Statistical analysis

Data are presented as means ± SE. Animal was recognized as a fixed effect with muscle as a random effect. All data were analyzed with a mixed model in JMP (SAS Institute Inc., Cary, NC). The least squares means were evaluated using a Student's t-test and considered significant at $P \leq 0.05$.

Results

MCT mRNA expression in porcine tissues

Primer efficiency was verified for MCT1, MCT2 and MCT4, and β-actin in pigs. Primer efficiency was between 95-102% for all primer sets and a single band was identified for each PCR product (Figure 5-1). MCT mRNA expression was quantified in a number of wild-type porcine tissues relative to β-actin. MCT1 expression was greatest in liver, kidney, and small intestines (Figure 5-2A). MCT2 expression was greatest in kidney, heart, and small intestines (Figure 5-2B). MCT4 expression was greatest in kidney, lung, small intestines and liver (Figure 5-2C). MCT1 and MCT2 mRNA expression was lower in the longissimus lumborum and masseter compared to other tissues. Relative MCT4 mRNA expression in the longissimus was intermediate to other tissues and greater than MCT1 and MCT2 expression.

MCT mRNA expression in porcine muscles

Relative MCT1, MCT2, and MCT4 mRNA expression was compared between the longissimus lumborum and masseter from wild-type and AMPK $\gamma3^{R200Q}$ pigs. Both MCT1 (Figure 5-3A) and MCT2 (Figure 5-3B) mRNA expression was greater ($P < 0.05$) in the masseter (oxidative muscle) than the longissimus lumborum (glycolytic muscle), but no genotype differences were detected in either muscle. MCT4 expression was greater ($P < 0.05$) in the longissimus lumborum than the masseter for both genotypes (Figure 5-3C). Furthermore, the longissimus lumborum from AMPK $\gamma3^{R200Q}$ pigs expressed greater ($P < 0.05$) MCT4 mRNA compared to the same muscle from wild-type pigs.

MCT protein expression in porcine muscles

Using SDS-PAGE and immunoblotting, MCT2 protein expression was detected in both the longissimus lumborum and masseter of both genotypes as a single band with an approximate molecular weight of 50 kDa (Figure 5-4A). The longissimus lumborum muscle contained lower ($P < 0.05$) MCT2 protein expression than the masseter in both genotypes (Figure 5-4B). Though, the longissimus lumborum of AMPK $\gamma3^{R200Q}$ pigs contained greater ($P < 0.05$) MCT2 protein abundance when compared to the same muscle from wild-type pigs. In order to confirm mRNA expression with protein data, we measured heart MCT2 in both genotypes. Independent of genotype, MCT2 protein expression was greater ($P < 0.05$) in the heart compared to the longissimus lumborum or masseter (Figure 5-5).

MCT4 was detected in the longissimus lumborum and masseter of both genotypes as a single band with an approximate molecular weight of 48 kDa (Figure 5-6A). The longissimus lumborum of AMPK $\gamma3^{R200Q}$ pigs contained greater ($P < 0.05$) MCT4 protein expression than all other muscle and genotype combinations (Figure 5-6B). No further differences were detected.

MCT protein abundance in porcine mitochondria

Solubilized proteins from the mitochondrial extractions of longissimus lumborum from wild-type and AMPK $\gamma3^{R200Q}$ pigs were first compared for sarcoplasmic or sarcolemma protein contamination. Glyceraldehyde 3-phosphate dehydrogenase (sarcoplasmic) and glucose transporter 4 (sarcolemma and sarcoplasmic) were detected in whole muscle

preparations, but not in the mitochondrial preparations of all mitochondrial extractions used (Figure 5-7A). The mitochondrial samples also contained an increased abundance of succinate dehydrogenase subunit A (mitochondrial protein) compared to whole muscle.

The mitochondrial extractions were analyzed for MCT2 and MCT4 protein abundance. A similarly sized protein as whole muscle MCT2 was detected from both wild-type and AMPK $\gamma3^{R200Q}$ pigs (Figure 5-7B). MCT4 was not detected in the mitochondrial extractions for either genotype (Figure 5-7C). MCT2 protein expression was not different (P = 0.23) between mitochondrial preparations between genotypes when normalized to equal loading of mitochondrial proteins (Figures 5-7B and 5-7D), though the AMPK $\gamma3^{R200Q}$ pigs were numerically greater.

MCT2 abundance of the mitochondrial was approximately 10 times greater than whole muscle when equal amount of total protein were separated by SDS-PAGE (Figure 5-8A). To account for differences in band density, we diluted the mitochondrial samples by 90% in order to further determine if the band identified in the whole muscle was consistent in size to the band from the mitochondria (Figure 5-8B and 7C). Once bands of similar density were produced, 50:50 mixtures from whole muscle and mitochondria preparations were created and separated using SDS-PAGE in an attempt to produce a doublet which would suggest molecular weight differences between the whole muscle and mitochondria MCT2 band. Though, only a single MCT2 band was identified in the mixed samples (Figure 5-8B) and this further suggests MCT2 is present in porcine skeletal muscle mitochondria.

Discussion

The original intention of this study was to determine the effect of chronic activation of AMPK on MCT protein expression. However, because data profiling mRNA expression of MCTs in pigs is limited and pigs are one of the few species who express MCT2, we measured MCT1, MCT2, and MCT4 mRNA expression from a number of tissues (Figure 5-2). Previous reports indicated that MCT1, MCT2 and MCT4 mRNA are all expressed in the small intestines and MCT1 and MCT4 are expressed in the colon (Parkunan, et al., 2015; Welter & Claus, 2008). Our data are consistent with these reports, but we have also determined that mRNA from all three MCTs tested is also expressed in kidney and liver (Figure 5-2). As with humans and rats, MCT isoform expression varies considerably between tissues based on physiological function (Bonen, Heynen, & Hatta, 2006).

We attempted to measure MCT1 protein expression in muscle, mitochondria, and all tissues used for gene expression with multiple antibodies. However, we were unable to detect MCT1 in any porcine tissue tested. A previous study detected MCT1 protein expression in heart and small intestines of pigs, but not in skeletal muscle (Sepponen, et al., 2003). Our mRNA expression data are consistent with those results as MCT1 mRNA expression in both the longissimus lumborum and masseter is low compared to other tissues (Figure 5-2A). MCT1 is generally expressed in most tissues, but especially those that import and oxidize lactate like liver and kidney (Halestrap, 2012). Based on our mRNA expression data, MCT1 protein may be expressed in many tissues. Because MCT1 expression is controlled by PGC-1α (Benton, et al., 2008; Hashimoto, et al., 2007) and chronic activation of AMPK increased PGC-1α expression (Garcia-Roves, et al., 2008),

further antibody testing or creation may be warranted for investigation of MCT1 expression in skeletal muscle.

MCT2 protein expression was present in all muscles tested and greater in the masseter (oxidative skeletal muscle) than the longissimus lumborum (glycolytic skeletal muscle) (Figure 5-4B). These data are consistent with the real-time PCR data (Figure 5-3B). Furthermore, MCT2 protein abundance was greater ($P < 0.05$) in the longissimus lumborum of AMPK $\gamma3^{R200Q}$ pigs compared to the longissimus lumborum of the wild-type pigs. Combined, these results show MCT2 expression is greater in red, oxidative muscles. However, these data do not agree with the previous study detailing MCT2 distribution in porcine skeletal muscle (Sepponen, et al., 2003). In it, the authors concluded that MCT2 was less abundant in oxidative muscles than glycolytic muscle. It is unclear why a discrepancy exists because both studies investigated MCT2 protein expression in the same muscles. However, the antibodies used in each study were different and this may explain the discrepancy. Porcine masseter contains predominantly oxidative, type I (~25%) and fast-oxidative, type IIA (~75%) muscle fibers (Realini, et al., 2013). The longissimus lumborum is comprised of predominantly of glycolytic type IIX and IIB fibers (Park, et al., 2009). Thus, the chosen muscles contain very different fiber-type profiles. Because both MCT1 and(or) MCT2 are thought to import lactate into red, oxidative muscle for oxidation (Halestrap, 2012), muscles with increased oxidative capacity should contain more MCT1 and(or) MCT2 (Bonen, 2001). Furthermore, our increased MCT2 mRNA expression (Figure 5-3B) in the masseter is consistent with the increased protein expression (Figure 5-4B). Similarly, the increased MCT2 protein expression in the longissimus lumborum of AMPK $\gamma3^{R200Q}$ pigs compared to wild-type may be due to the increased

oxidative capacity of the muscle (Scheffler, et al. 2014). In an attempt to further address the conflict, we determined MCT2 protein abundance in heart. Based on the increased MCT2 mRNA expression from the heart compared to the masseter (Figure 5-4B) and that the heart contains substantially more mitochondria, MCT2 protein expression should also be greater. As expected, the heart expressed greater MCT2 protein compared to the longissimus lumborum and masseter independent of genotype (Figure 5-5). Thus, MCT2 is more abundant in porcine muscle with greater oxidative capacity.

Recent evidence has localized MCT1 and MCT2 to subsarcolemmal and intermyofibrillar mitochondria from skeletal muscle (Benton, et al., 2004; Brooks, Brown, Butz, Sicurello, & Dubouchaud, 1999). Because the magnitude of difference between mitochondrial number (~50%) from our previous study (Scheffler, et al., 2014) was similar to the MCT2 protein expression difference betwee the longissimus lumborum from AMPK $\gamma3^{R200Q}$ and wild-type pigs, we investigated if MCT2 was present in the mitochondria. Because we did not expect to provide evidence that MCTs are present in the mitochondria, we did not separate the two sub-populations of mitochondria. Though, based on our extraction techniques, the majority of the mitochondria in our extracts are likely subsarcolemmal mitochondria (Benton, et al., 2004; Campbell, et al., 2004). Regardless, we were able to detect MCT2 in our mitochondrial extractions that were highly purified and free from contamination by sarcoplasmic or sarcolemmal proteins (Figure 5-7A). While it is possible individual mitochondria from the AMPK $\gamma3^{R200Q}$ pigs contains a greater number of MCT2 molecules per mitochondria, we were unable to detect a difference for mitochondria MCT2 between genotypes (Figure 5-7B and 5-7D). Furthermore, the metabolic demand of red, oxidative muscle is met by increasing the number of

mitochondria as opposed to altering the actual mitochondrial protein composition (Glancy & Balaban, 2011). Thus, MCT2 is present in the mitochondria of pigs and this further suggests MCT2 expression is greater in oxidative muscles. Though immunohistochemical co-localization to the mitochondrial membranes is warranted for further confirmation.

We concede, however, that the increased in MCT2 protein expression from the masseter of either genotype or the longissimus lumborum from AMPK $\gamma3^{R200Q}$ pigs may not be due to increased mitochondria content. Rather, it is possible we may have inadvertently purified the sarcolemma or peroxisomes containing MCT2 (Hashimoto, Masuda, Taguchi, & Brooks, 2005). While our data suggest otherwise (Figure 5-7A), an increase in sarcolemmal MCT2 alone in whole muscle is still consistent with previous reports. Increased oxidative capacity of the masseter and the longissimus lumborum of the AMPK $\gamma3^{R200Q}$ pigs can still explain the increased protein expression due to the intercellular lactate shuttle. Lactate is shuttled intercellularly into red, oxidative muscle fibers by MCT1 or MCT2 from white, glycolytic muscle fibers (Brooks, 2002). All muscles exhibiting greater MCT2 protein expression in this study are associated with a more oxidative muscle phenotype than the longissimus lumborum from wild-type pigs (Lebret, et al., 1999; Realini, et al., 2013). Thus, the MCT2 protein expression data are still consistent with previous reports even if the mitochondrial data are in error.

Our mitochondrial MCT2 protein expression data add to the ongoing debate of whether MCTs are truly localized to the mitochondria. MCT1, MCT2 or MCT4 have all been detected in mitochondria of skeletal muscle (Briand, Talmant, Briand, Monin, & Durand, 1981; Brooks, et al., 1999; Butz, McClelland, & Brooks, 2004; Hashimoto, et al., 2005). Furthermore, MCT1 was identified in the mitochondria of porcine large intestines

(Welter & Claus, 2008). Yet, others have refuted these claims (Halestrap, 2012; Hashimoto, et al., 2005). Specifically, the controversy arises about whether lactate is oxidized intracellularly, intercellularly, or both. In essence, the theory of intracellular transport claims that lactate produced under anaerobic conditions is transported into the mitochondria of the same cell where a mitochondrial-based lactate dehydrogenase converts it to pyruvate for mitochondrial oxidation. Evidence for and against the intracellular lactate shuttle theory is available in a number of review articles (Brooks, 2009; Halestrap & Wilson, 2012; Sahlin, Fernström, Svensson, & Tonkonogi, 2002). Even if the intracellular lactate shuttle theory is inaccurate, MCTs may still exist in mitochondria as a pyruvate transporter due to its role as a high affinity pyruvate transporter (Benton, et al., 2004). However, MCT2 is not believed to be the highly sought after, but elusive mitochondrial pyruvate carrier (Bricker, et al., 2012; Hildyard & Halestrap, 2003).

MCT4 protein was detected in both skeletal muscles and genotypes. No differences in protein abundance were detected between the longissimus lumborum and masseter of the wild-type pigs. These data are consistent with previous determination of MCT4 abundance in porcine skeletal muscle (Sepponen, et al., 2003). However, MCT4 expression (Figure 5-3C) and protein abundance (Figure 5-6B) was greater in the longissimus lumborum of AMPK $\gamma3^{R200Q}$ pigs. Three mechanisms for MCT4 expression regulation have been proposed in skeletal muscle. Under hypoxic conditions, transcriptional regulation by hypoxia inducible factor 1α (HIF-1α) upregulated MCT4 expression (Ullah, Davies, & Halestrap, 2006). Alternatively, clenbuterol, a β2-adrenergic agonist, treatment increased both the receptor interacting protein 140 (RIP140) and MCT4 protein abundance in skeletal muscle (Hoshino, et al., 2012). RIP140 contributes to the increase of glycolytic

muscle fibers and decrease in mitochondrial content (Seth, et al., 2007). Neither mechanism, though, has been directly connected to AMPK activation (Hamada & Takimoto, 2013). Our data suggest AMPK activation can directly increase MCT4 expression in skeletal muscle independent of either proposed mechanism. This conclusion is consistent with previous reports indicating increased AMPK activation increases MCT4 expression in skeletal muscle (Furugen, et al., 2011; Hamada & Takimoto, 2013; Thomas, et al., 2012).

Explaining the purpose for the simultaneous increase in MCT2 and MCT4 in the longissimus lumborum of AMPK $\gamma3^{R200Q}$ pigs is still unclear. Typically, these transporters serve opposite functions and a simultaneous increase in expression is unlikely in any tissue (Halestrap & Wilson, 2012). MCT4 normally functions to export lactate from a cell while MCT1 and MCT2 are thought to import lactate into a cell for oxidation. Yet, acute AMPK activation simultaneously increased MCT1 and MCT4 expression in glycolytic muscle of rats (Takimoto, Takeyama, & Hamada, 2013). Therefore, increasing both transporters is advantageous to increase energy producing pathways. The increased MCT2 expression may contribute to an increased ability for pyruvate uptake by the mitochondria. Extracted mitochondria from AMPK $\gamma3^{R200Q}$ pigs displayed enhanced state 3 respiration when pyruvate was used as a substrate (Scheffler, et al., 2014). The increased MCT4 expression may be in response to the increased lactate produced by the glycolytic longissimus lumborum. AMPK $\gamma3^{R200Q}$ pigs contain lower resting lactate in the longissimus lumborum (unpublished data) and the greater MCT4 abundance may be responsible for the decrease.

Conclusions

In summary, chronic activation of AMPK increases MCT2 and MCT4 abundance in glycolytic porcine skeletal muscle. To simultaneously increase both MCT2 and MCT4 suggests regulation may be independent of each other, but still connected to the metabolic demands of the muscle. However, the exact signaling mechanisms responsible for the increased MCT4 expression are still unclear while the mechanism for MCT2 expression are unknown (Hamada & Takimoto, 2013). To our knowledge, this study is the first to identify a connection between AMPK activation and MCT2 expression in any tissue.

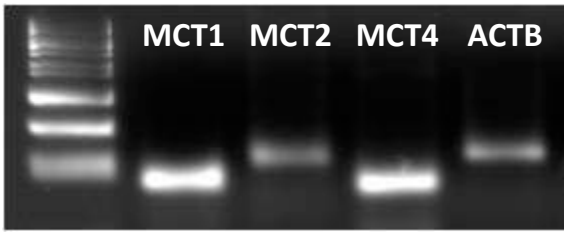
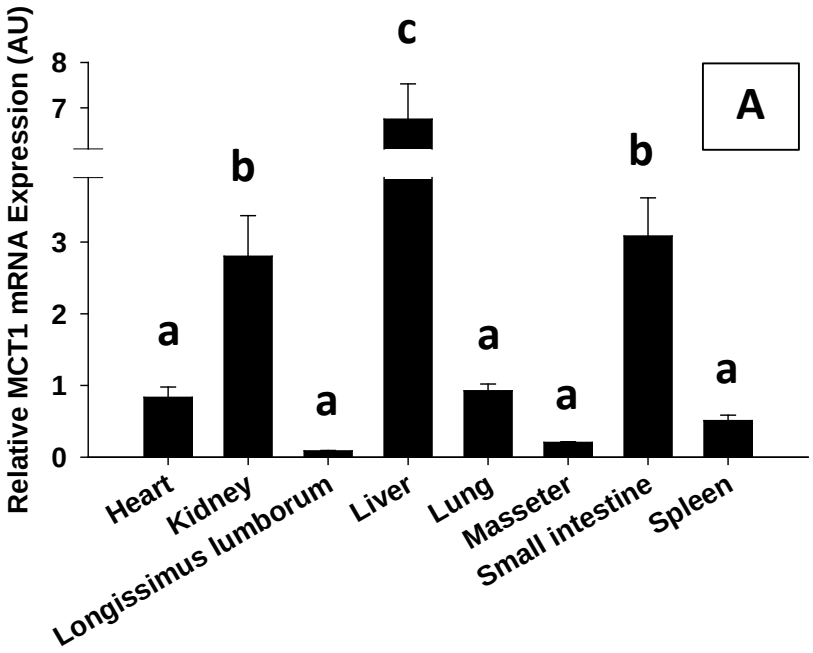
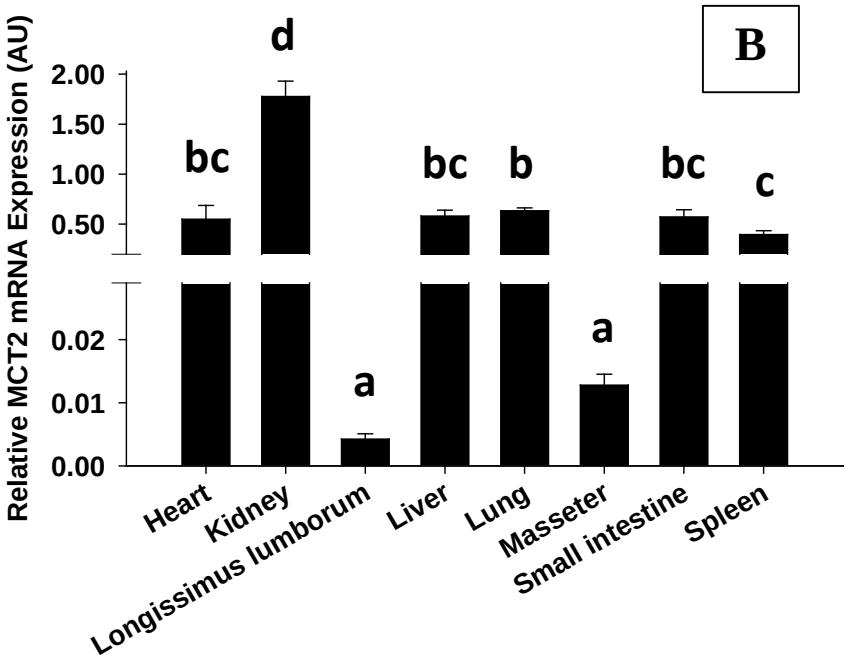


Figure 5-1. Representative real-time quantitative polymerase chain reaction product separated on a 2% agarose gel. MCT1: Monocarboxylate transporter 1. MCT2: Monocarboxylate transporter 2. MCT4: Monocarboxylate transporter 4. ACTB: β-actin.


A
Relative MCT1 mRNA Expression (AU)
8
7
3
2
1
0
a
b
a
c
a
a
b
a
Heart
Kidney
Longissimus lumborum
Liver
Lung
Masseter
Small intestine
Spleen


B
Relative MCT2 mRNA Expression (AU)
2.00
1.50
1.00
0.50
0.02
0.01
0.00
bc
d
a
bc
b
a
bc
c
Heart
Kidney
Longissimus lumborum
Liver
Lung
Masseter
Small intestine
Spleen

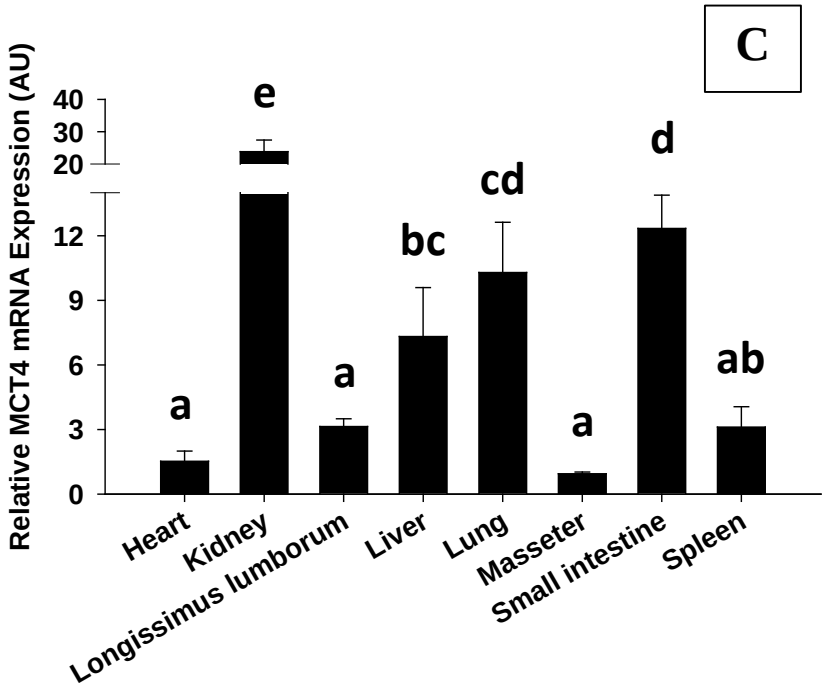
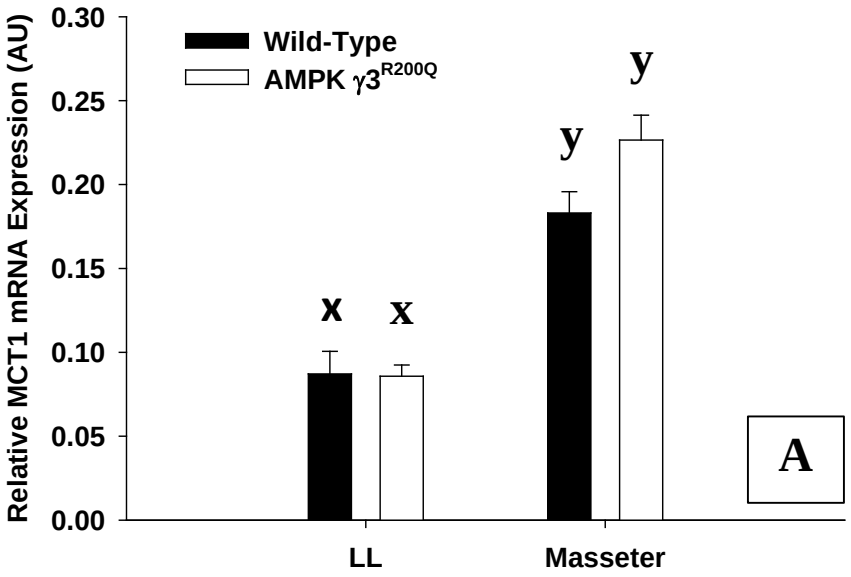
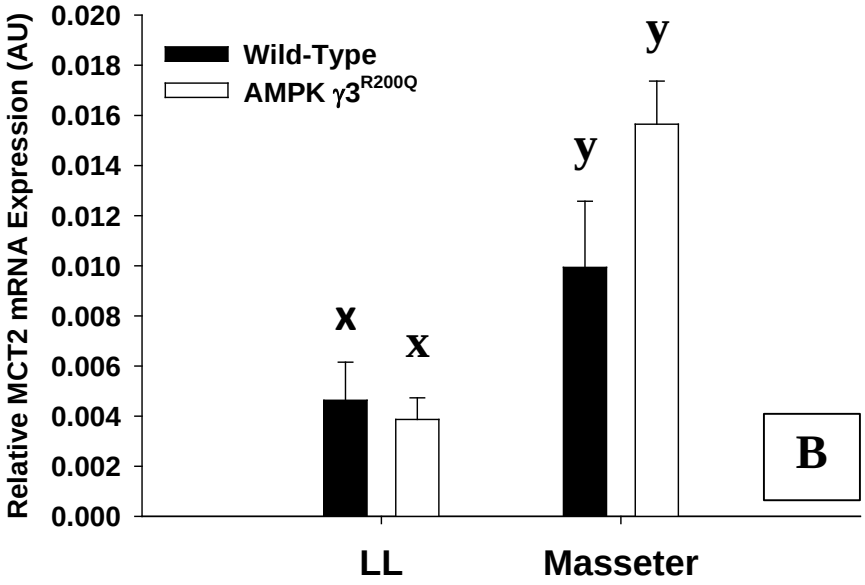


Figure 5-2. Mean relative MCT1 (A), MCT2 (B), and MCT4 (C) mRNA expression of porcine tissues from wild-type pigs (n = 4-6 per tissue).


Relative MCT1 mRNA Expression (AU)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Wild-Type
AMPK γ3R200Q
x
x
y
y
A
LL
Masseter


Relative MCT2 mRNA Expression (AU)
0.020
0.018
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
Wild-Type
AMPK γ3R200Q
x
x
y
y
B
LL
Masseter

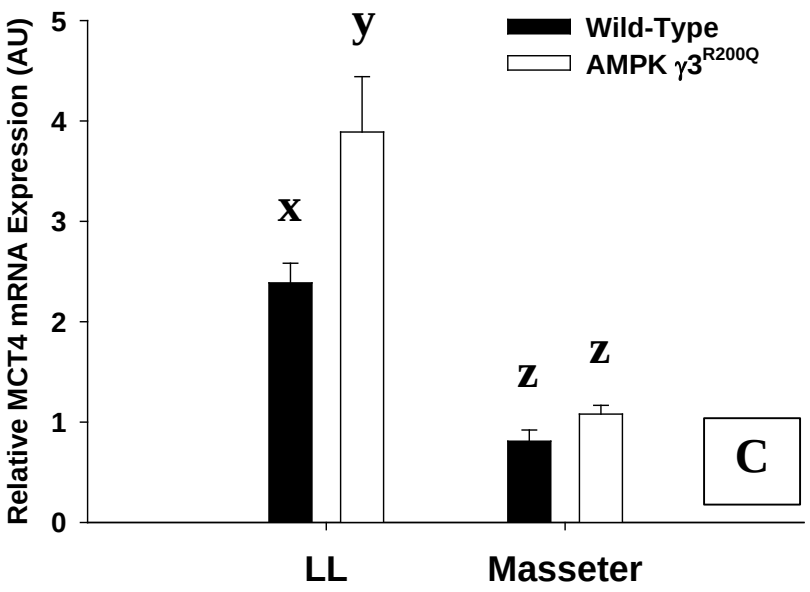


Figure 5-3. Mean relative MCT1 (A), MCT2 (B), and MCT4 (C) mRNA expression of longissimus lumborum (LL) and masseter muscles from wild-type and AMPK $\gamma3^{R200Q}$ pigs (n = 6 per muscle and genotype combination). Means without a common superscript for $MCT1^{x-y}$, $MCT2^{x-y}$, or $MCT4^{x-z}$ are different ($P < 0.05$).

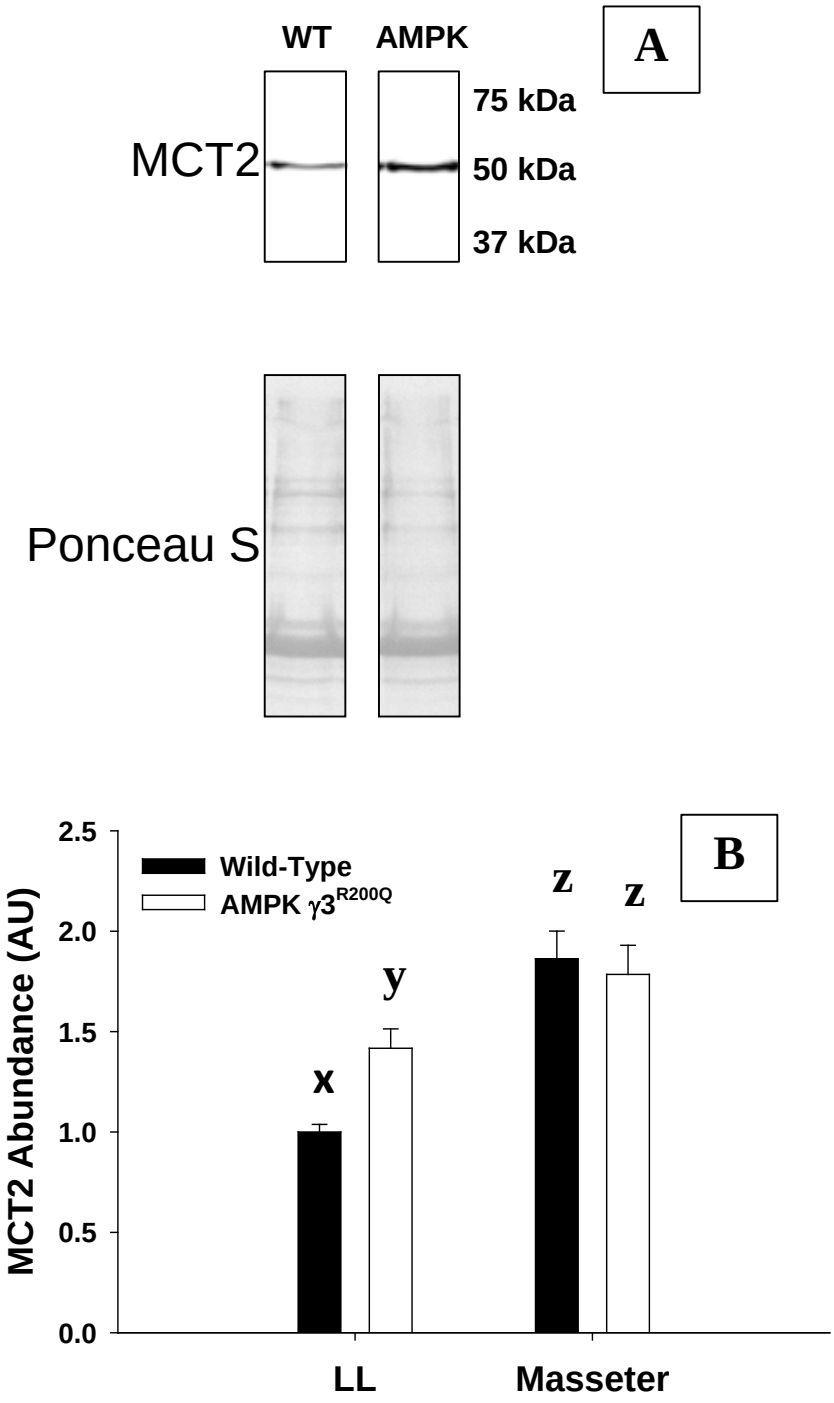


Figure 5-4. (A) Representative immunoblot of MCT2 from the longissimus lumborum and masseter from wild-type (WT) A: Representative immunoblot ofMCT2 from the longissimus lumborum and masseter from wild-type (WT) and AMPK $\gamma3^{R200Q}$ (RN^{-}) pigs. (B) Mean MCT2 protein abundance from the longissimus lumborum (LL) and masseter muscles from wild-type and AMPK $\gamma3^{R200Q}$ mutant pigs (n = 6 per muscle and genotype). Means without a common superscript for $MCT2^{x-z}$ are different ($P < 0.05$).

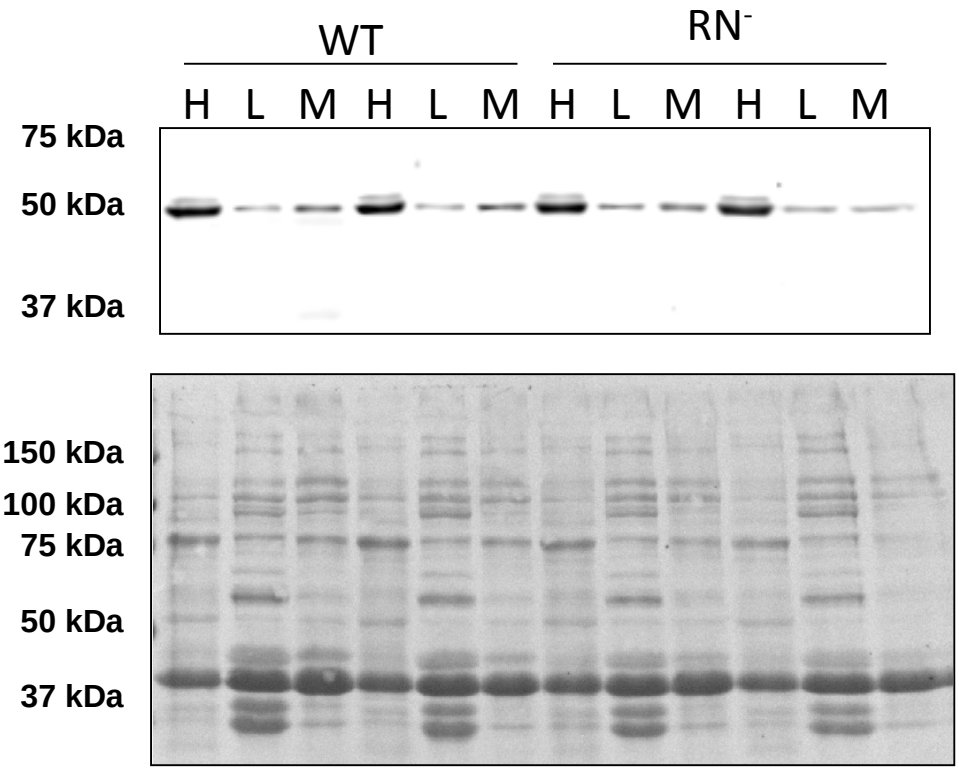


Figure 5-5. Immunoblot of MCT2 from the heart (H), longissimus lumborum (L) and masseter (M) from wild-type (WT) and AMPK $\gamma3^{R200Q}$ (RN^-) pigs.

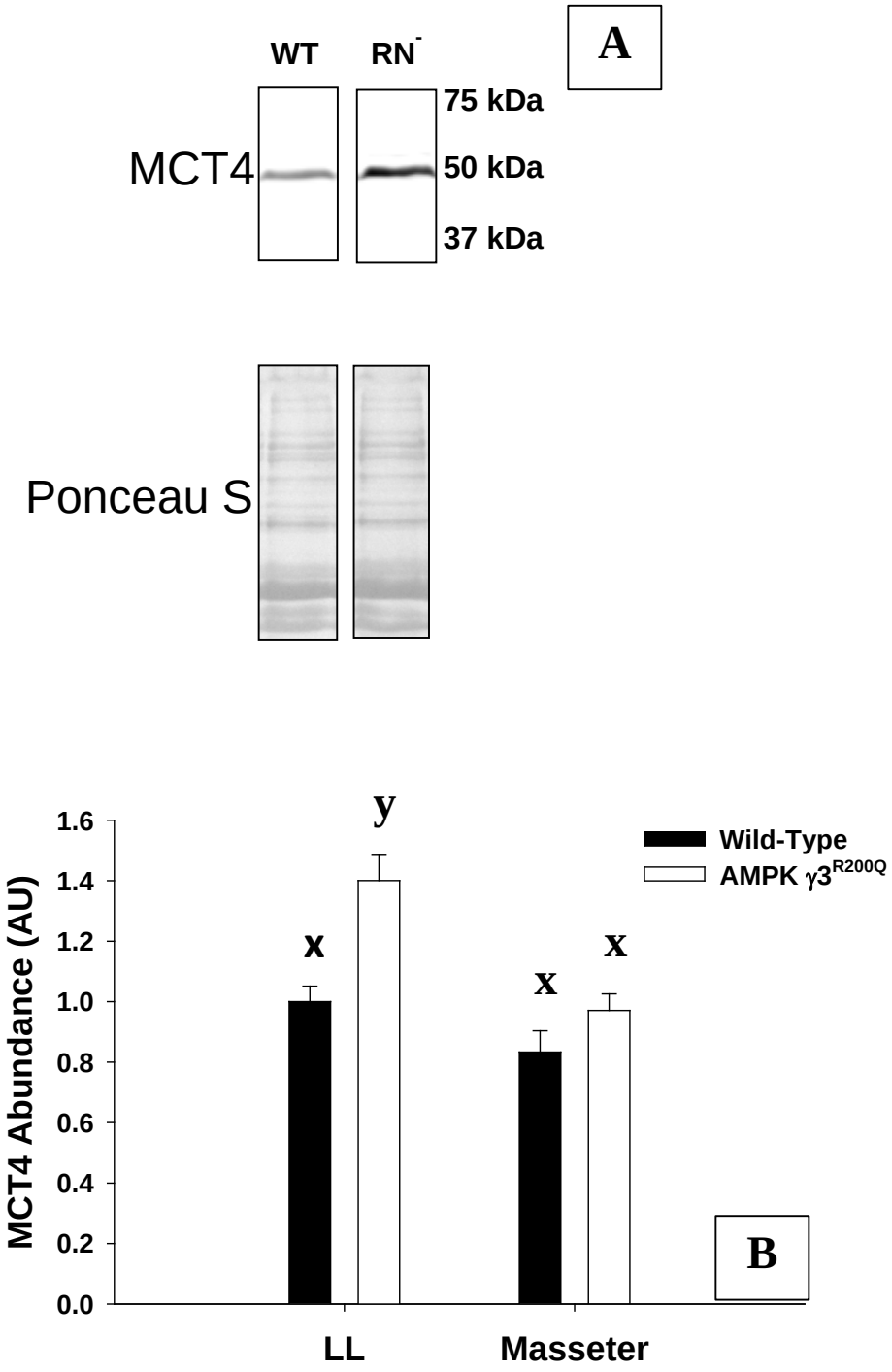
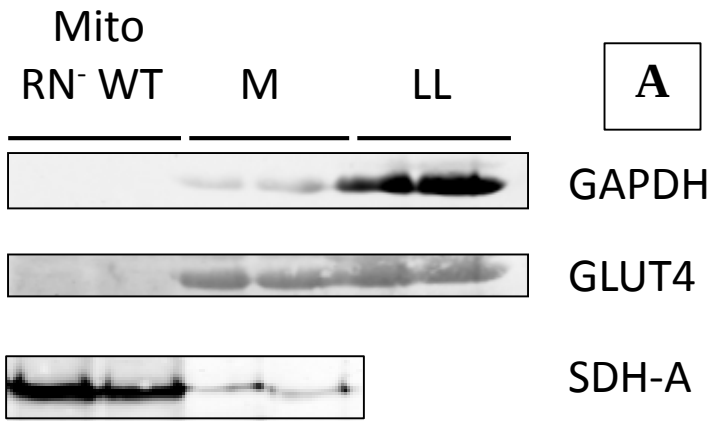
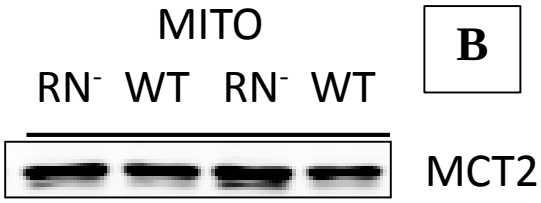


Figure 5-6. (A) Representative immunoblot of MCT4 from the longissimus lumborum and masseter from wild-type (WT) and AMPK $\gamma3^{R200Q}$ (RN^-) pigs (n = 6 per muscle and genotype). (B) Mean MCT4 protein abundance from the longissimus lumborum (LL) and masseter muscles from wild-type and AMPK $\gamma3^{R200Q}$ mutant pigs. Means without a common superscript for $MCT4^{x-y}$ are different ($P < 0.05$).


Mito
RN- WT
M
LL
A
GAPDH
GLUT4
SDH-A


MITO
RN- WT RN- WT
B
MCT2


Mito
RN- WT
LD
M
C
MCT4

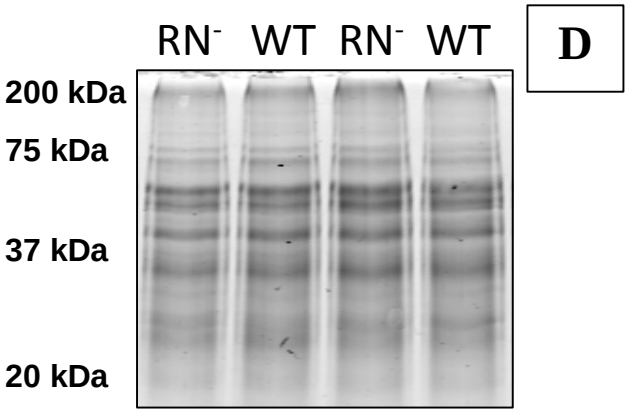

RN- WT RN- WT
D
200 kDa
75 kDa
37 kDa
20 kDa

Figure 5-7. (A) Representative immunoblot of mitochondrial extraction sample. Antibodies specific for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), glucose transporter 4 (GLUT4), and succinate dehydrogenase subunit A (SDH-A) were used to assess contamination (GAPDH, GLUT4) or mitochondrial extraction efficiency (SDH-A). (B) Representative immunoblot of MCT2 from the longissimus lumborum (wild-type), masseter (wild-type), or longissimus lumborum mitochondrial extractions from wild-type (WT) or AMPK $\gamma3^{R200Q}$ (RN^-) pigs (n = 4 per genotype). (C) Representative immunoblot of MCT4 from the longissimus lumborum (wild-type), masseter (wild-type), or longissimus lumborum mitochondrial extractions from wild-type (WT) or AMPK $\gamma3^{R200Q}$ (RN^-) pigs (n = 4 per genotype). (D) Representative ponceau S stained blot from longissimus lumborum mitochondrial extractions from wild-type (WT) or AMPK $\gamma3^{R200Q}$ (RN^-) pigs.

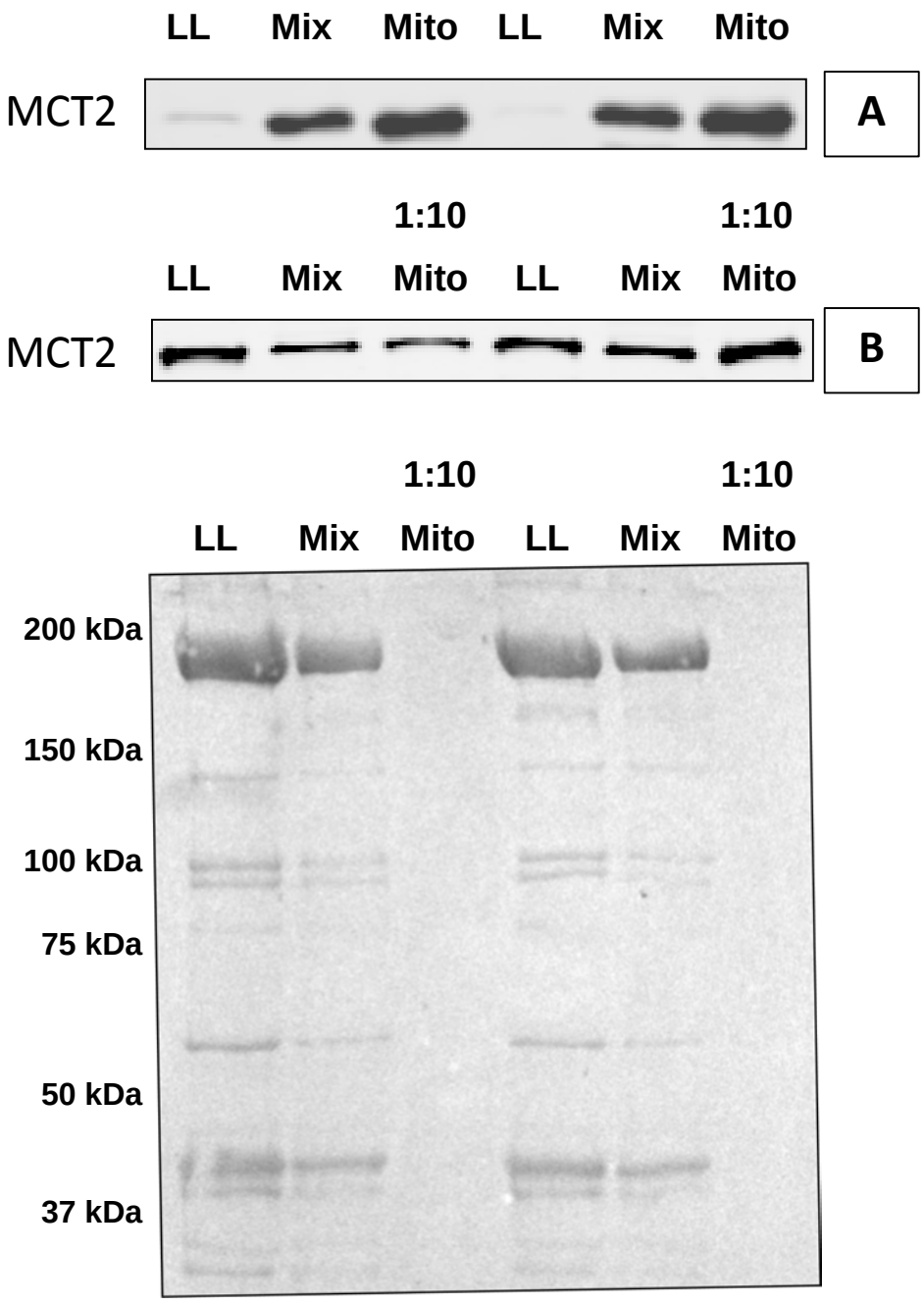


Figure 5-8. (A) Representative immunoblot of longissimus lumborum whole muscle (LL), extracted mitochondria (Mito), or a 50:50 mixture of the two samples. (B) Representative immunoblot of longissimus lumborum whole muscle (LL), extracted mitochondria diluted by 90% (Mito), or a 50:50 mixture of the two samples with ponceau S staining used as a loading control.

Table 5-1. Nucleotide sequences of the primers used for quantitative real-time PCR.

		Primer Sequences	Accession No.
β-Actin	Forward:	CCAGCACCATGAAGATCAAGATC	XM_003357928.2
	Reverse:	ACATCTGCTGGAAGGTGGACA	
MCT1	Forward:	ATTCTTGGGGGCTTGCTGTT	NM_001128445.1
	Reverse:	TTGTCTGCAGTGGTTGGCTT	
MCT2	Forward:	TGGGAATGGTCACAGCTTCC	XM_005663944.1
	Reverse:	GGGCTGCAGGTTAAAGGCTA	
MCT4	Forward:	CATCACGGGCTTCTCCTACG	XM_003357925.2
	Reverse:	AGCCGATGCCGAACTCAC	

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