Phosphofructokinase and mitochondria partially explain the high ultimate pH of broiler pectoralis major muscle

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ABSTRACT During postmortem metabolism, muscle pH gradually declines to reach an ultimate pH near 5.6 across most meat species. Yet, broiler pectoralis major (P. major) muscle generates meat with high ultimate pH (pH $\sim$ 5.9). For better understanding of the underlying mechanism responsible for this phenomenon, we evaluated the involvement of breast muscle chilling on the extent of postmortem metabolism. Broiler breast muscles were either subjected to chilling treatment (control) or left at room temperature (RT) for 120 min. P. major muscle from the RT treatment had lower ultimate pH, greater glycogen degradation and lactate accumulation. While these findings suggest that carcass chilling can contribute to the premature termination of postmortem metabolism, chilling did not fully explain the high ultimate pH of P. major muscle. Our results also revealed that glucose-6-phosphate (G6P) was very low at 24 h, and therefore we hypothesized that G6P was limiting. To test this hypothesis, muscle samples from P. major and porcine longissimus lumborum (LL) muscle were homogenized into a reaction buffer that mimics postmortem glycolysis with or without 0.5 mg/mL isolated mitochondria. While samples containing porcine LL muscle reached the normal level of ultimate pH, P. major muscle samples reached a value similar to that observed in vivo even in the presence of excess G6P, indicating that G6P was not limiting. Mitochondria enhanced the glycolytic flux and pH decline in systems containing muscle from both species. More importantly, however, was that in vitro system containing chicken with mitochondria reached pH value similar to that of samples containing LL muscle without mitochondria. To investigate further, phosphofructokinase (PFK) activity was compared in broiler P. major and porcine LL muscle at different pH values. PFK activity was lower in P. major muscle at pH 7, 6.5, and 6.2 than LL muscle. In conclusion, carcass chilling can partially contribute to the high ultimate pH of broiler P. major muscle, while low PFK activity and mitochondria content limit the flux through glycolysis.

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

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INTRODUCTION

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

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as firmness, dryness, and short shelf life may still exist (Allen et al., 1997).

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

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

MATERIALS AND METHOD

Bird Slaughter and Muscle Sampling

All experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee at Virginia Tech. A total of 30 broiler chickens (mixed sex, 42 days of age, 3.0 ± 0.04 kg body weight) were obtained from the Virginia Tech Poultry Research Facility. Following a 10 h feed withdrawal period, all birds were harvested at the same facility using standard commercial procedures. Birds were stunned with carbon dioxide and immediately exsanguinated by severing both carotid arteries and at least one jugular vein. After bleeding for 120 s, skin was removed from the cranial part of the right pectoralis major (P. major) muscle and approximately 2 cm^3 sample was collected

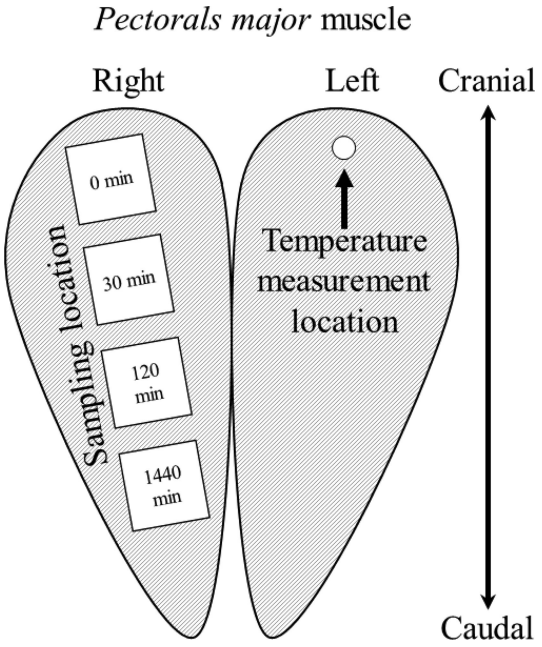

Figure 1. Schematic diagram of muscle sampling lactations and temperature measurements.

(subsequently referred to as 0 min sample; Figure 1). Samples were immediately snap frozen in liquid nitrogen, and stored at −80°C. Following, birds were scalded at 60°C for 90 s, defeathered in a rotary drum picker for 30 s, and manually eviscerated. Immediately after evisceration, whole breast muscles were removed from each carcass, labeled, and assigned to one of 2 chilling treatments (n = 15 per treatment). Treatments were: chilling in ice water at 0.5°C for 120 min (Control) or held at room temperature (**RT**) for 120 min. Following, muscles from both treatments were placed in cold storage room at 4°C until 24 h postmortem. Additional muscle samples were collected and stored in the same manner as described for the 0 min samples at 30, 120, and 1,440 min (24 h) postmortem from the right P. major muscle (Figure 1).

Temperature

Muscle internal temperature was measured at 10, 30, 60, 120, 240, and 1,440 min postmortem through an incision made by a knife in the cranial part of the left P. major muscle (Figure 1) using a data logger thermometer (HH147U; Omega Engineering, Inc., Norwalk, CT).

Pectoralis Major Muscle pH and Metabolite Analysis

Frozen 0, 30, 120, and 1,440 min P. major muscle samples were powdered under liquid nitrogen using a mortar and pestle, and 3 tubes of approximately 0.1 g were collected. For pH analysis, powdered muscle samples were lysed using a Tissue Lyser II system (Qiagen, Boston, MA) in 0.8 mL of ice-cold solution containing 5 mM sodium iodoacetate and 150 mM KCl

(pH 7.0) (Bendall, 1973). Samples were then centrifuged at 17,000 × *g* for 5 min, equilibrated to 25°C, and measured directly using an Orion Ross Ultra pH glass electrode (Thermo Scientific, Pittsburgh, PA). Samples designated for glucose, glucose-6-phosphate (**G6P**), lactate, adenine nucleotides, and inosine monophosphate (**IMP**) analysis were lysed in 1 mL of ice-cold 0.5 M perchloric acid. After incubating on ice for 20 min, homogenates were centrifuged at 17,000 × *g* for 5 min, and the resulting supernatants were transferred to new tubes and neutralized with 2 M KOH (Bergmeyer, 1984). For muscle glycogen determination, another sample was lysed in 1 mL of 1.25 M HCl, heated at 90°C for 2 h, and centrifuged at 17,000 × *g* for 5 min. Supernatants were transferred to new tubes and neutralized with 1.25 M KOH (Bergmeyer, 1984). Glycogen, glucose, G6P, and lactate were determined using enzymatic methods modified for a 96-well plate as described by (Hammelman et al., 2003). Adenine nucleotide and IMP contents were quantified using HP Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA) and external standards (Bernocchi et al., 1994; Williams et al., 2008).

Porcine Muscle Sampling

Market-weight pigs (n = 6) were slaughtered in the Virginia Tech Meat Center using standard commercial procedures. Muscle samples were excised from the longissimus lumborum (**LL**) muscle at 0 min (within 5 min of exsanguination) postmortem. Samples were used for mitochondrial extraction or immediately snap frozen in liquid nitrogen, and stored at −80°C.

Mitochondria Isolation

Mitochondria were isolated from porcine LL muscle by differential centrifugation according to (Scheffler et al., 2015). Briefly, muscle samples were finely minced with scissors in ice-cold isolation buffer (5 mL/g of muscle; 100 mM sucrose, 180 mM KCl, 50 mM Tris, 5 mM $MgCl_2$, 10 mM EDTA, 1 mM K-ATP, pH 7.4). Protease (subtilisin A) was added to the tissue suspension at 0.4 mg/mL followed by homogenization with a Potter-Elvehjem type homogenizer system (Glas-Col, Terre Haute, IN). Homogenates were diluted with isolation buffer to achieve ∼ 20 mL/g of muscle before filtering through 2 layers of cheese-cloth. Homogenates were then centrifuged at 1,000 × *g* for 10 min at 4°C followed by a second filtration of the supernatant through cheese-cloth. Filtered supernatants were centrifuged again at 8,000 × *g* for 10 min at 4°C. Resulting mitochondrial pellets were suspended in mitochondrial suspension buffer (220 mM mannitol, 70 mM sucrose, 10 mM Tris–HCl, and 1 mM EGTA, pH 7.4). Mitochondrial protein concentration was determined using a bicinchoninic acid (**BCA**) protein assay kit (Pierce, Rockford, IL).

In Vitro Glycolysis Model

Frozen 0 min P. major (n = 6) and porcine LL (n = 6) muscles were pulverized under liquid nitrogen and homogenized at 1:10 (wt/vol) in reaction buffer containing 40 mM glycogen, 60 mM KCl, 5 mM $MgCl_2$, 10 mM Na_2HPO_4, 30 mM creatine, 25 mM carnosine, 10 mM sodium acetate, 5 mM ATP, 0.5 mM ADP, and 0.5 mM NAD^+ (pH 7.4) (England et al., 2014). Either 0 or 0.5 mg/mL isolated mitochondria were incorporated into the in vitro model. Aliquots were removed at 0, 30, 120, 240, and 1,440 min for pH and metabolite analysis. Reaction vessels were maintained at 25°C for the duration of the trial.

In vitro pH and Metabolite Analysis

Aliquots for pH analysis were mixed with 25 mM sodium iodoacetate and 750 mM KCl solution (pH 7.0) at 4:1 (vol/vol). Samples were then centrifuged at 17,000 × *g* for 5 min at room temperature, equilibrated to 25°C, and measured directly using an Orion Ross Ultra pH glass electrode (Thermo Scientific, Pittsburgh, PA). Samples for glucose, G6P, and lactate determination were added to ice-cold 1 M perchloric acid at 1:1 ration. After incubating on ice for 20 min, samples were centrifuged at 17,000 × *g* for 5 min, and the resulting supernatants were neutralized with 2 M KOH. Aliquots for glycogen analysis were mixed with equal volume of 2.5 M HCl, heated at 90°C for 2 h, centrifuged at 17,000 × *g* for 5 min. Supernatants were transferred to new tubes and neutralized with 1.25 M KOH. Glycogen, glucose, G6P, and lactate were measured according to (Hammelman et al., 2003).

Phosphofructokinase Activity Assay

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

Statistical Analysis

Effects of chilling treatment and time on metabolites and pH were analyzed as a split-plot design using the

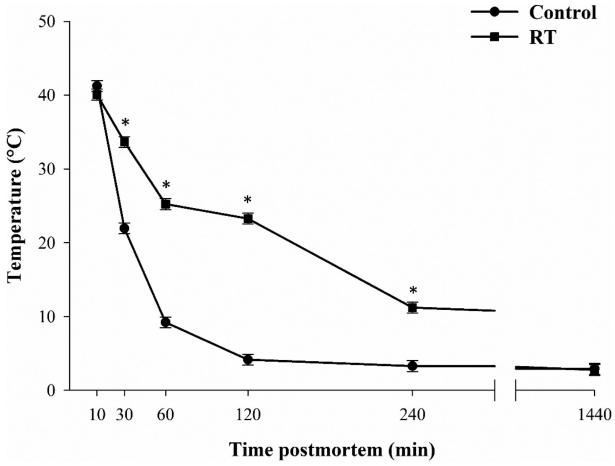

Figure 2. Broiler P. major muscle temperature (°C) of control and RT treatments. Data are LS means ± SE. *indicates significant difference within a time point ($P < 0.05$).

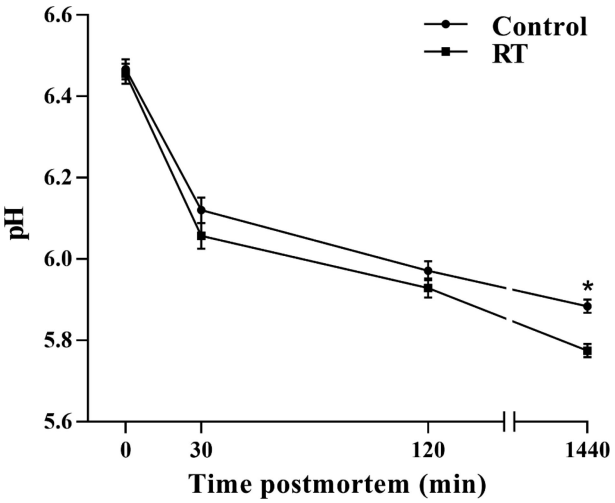

Figure 3. Mean pH in broiler P. major muscle of control and RT treatments. Data are LS means ± SE. *indicates significant difference within a time point ($P < 0.05$).

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

RESULTS AND DISCUSSION

Temperature and pH

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

The chilling treatment significantly influenced pH decline of the P. major muscle ($P = 0.02$; Figure 3). While no differences were detected through 120 min postmortem, a lower ultimate pH (at 1,440 min, $P = 0.02$) was observed in muscles from the RT treatment (5.8 ± 0.01) compared to control (5.9 ± 0.01). These findings are in agreement with previously published reports (McKee and Sams, 1998; Alvarado and Sams, 2002; Öztürk and Serdaroglu, 2015), where higher carcass temperatures were associated with lower ultimate pH. However, breast muscle from the RT treatment still possesses a greater than normal ultimate pH (5.5 to 5.7), suggesting other mechanisms may be involved in determining the extent of postmortem pH decline of broiler breast muscle. Results of this study nonetheless suggest that carcass chilling rate can modulate the extent of pH decline in broiler breast and may account for the differences in ultimate pH usually observed between different species.

Glycolytic Metabolites and Adenine Nucleotides

For better understanding of postmortem metabolism, glycolytic metabolite and adenine nucleotide levels were measured in the P. major muscle. During the postmortem period, pyruvate generated through glycolysis is converted to lactate and accumulates in the muscle. As expected, patterns of lactate formation in the current study followed pH decline. Lactate concentration of the P. major muscle was not affected by the chilling treatment through 120 min. However, at 1,440 min, muscles from the RT treatment had significantly greater lactate ($P = 0.01$; Figure 4A) when compared to that of control. These results confirm that lower ultimate pH observed in the RT treatment was due to greater flux through the glycolytic pathway. It is well established that rapid cooling of carcasses slows metabolic enzyme activity and limits rate of metabolism (Bowker et al., 2000). Bock and Frieden (1974) reported that PFK loses as much as 97% of its activity as temperature reduces from 20°C to 3°C. The authors suggested that lower temperature enhances enzyme dissociation

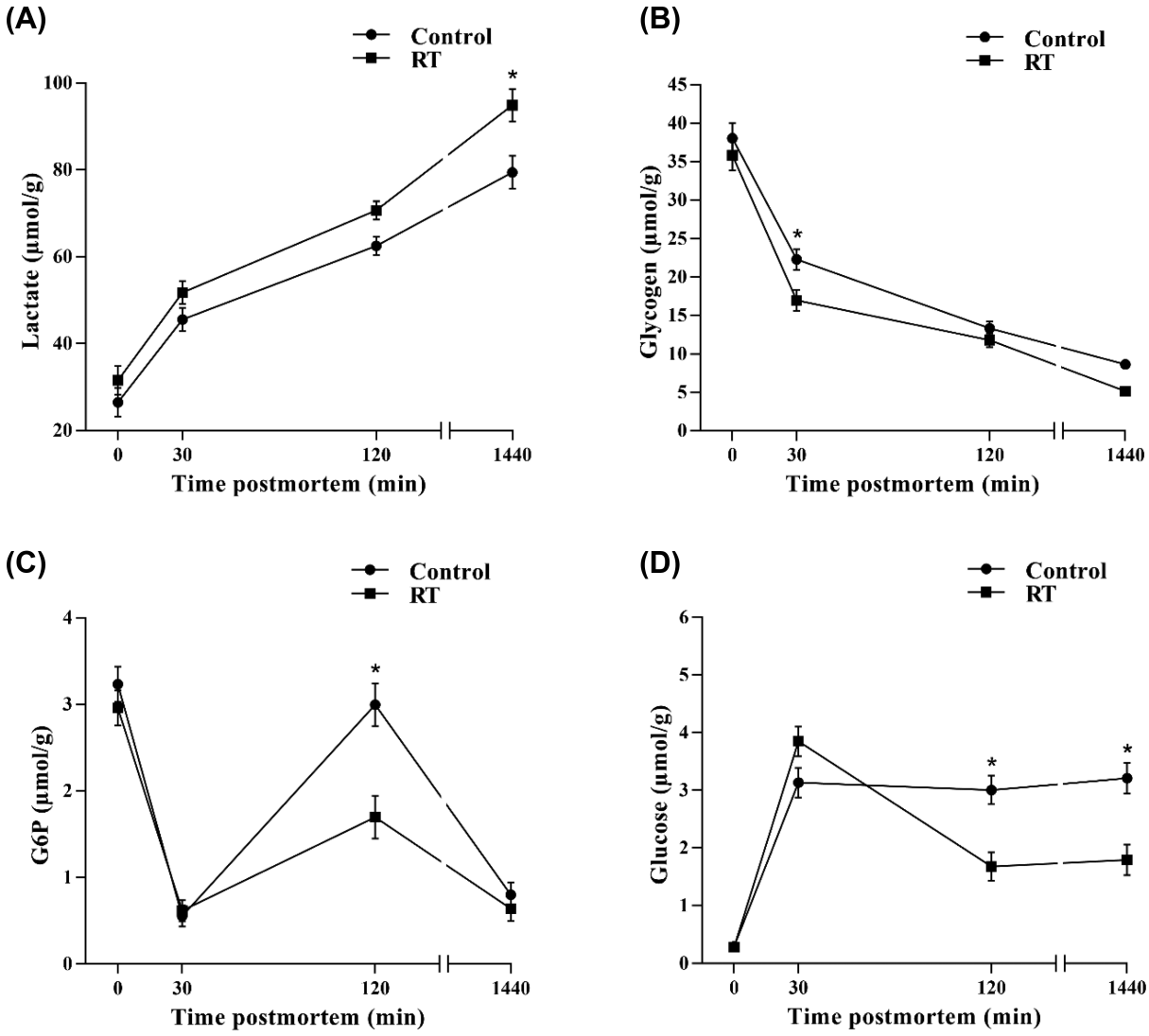

Figure 4. Mean lactate (A; μmol/g), glycogen (B, μmol/g), G6P (C; μmol/g) and glucose (D; μmol/g) in broiler P. major muscle of control and RT treatments. Data are LS means ± SE. *indicates significant difference within a time point ($P < 0.05$).

from its more active tetrameric form to a less active dimeric form. This in turn reduces glycolytic flux, resulting in high ultimate pH (England et al., 2014).

Mobilization of muscle glycogen during postmortem metabolism drives pH decline. Therefore, inadequate muscle glycogen can lead to premature cessation of postmortem metabolism (Henckel et al., 2002). Glycogen degradation yields glucose 1-phosphate (**G1P**) and non-phosphorylated glucose molecules. Subsequently, G1P is isomerized by phosphoglucomutase to G6P and enters the glycolytic pathway, while free glucose molecules are either converted by hexokinase to G6P or accumulated in postmortem muscle (Young et al., 1988; Scheffler and Gerrard, 2007). At 30 min postmortem, glycogen was lower in samples from the RT treatment compared to controls ($P = 0.04$; Figure 4B). However, no differences in glycogen among treatments were observed at 120 and 1,440 min. The overall mean glycogen concentration at 0 min was 37 ± 1.5 μmol/g of muscle; a concentration lower than those found in LL muscle of pork (Copenhafer et al., 2006; Matarneh et al., 2015) and beef (Frylinck et al., 2013; Apaoblaza et al., 2015). The low glycogen concentration in pectoralis muscle of current commercial broilers has been attributed to the intensive selection for increased breast yield (Berri et al., 2005; Le Bihan-Duval et al., 2008). Even so, however, glycogen was not depleted in either treatment at 1,440 min, indicating that glycogen was not limiting and postmortem glycolysis could have continued. The impact of chilling treatment on G6P was dependent on time (treatment × time, $P = 0.03$, Figure 4C). While no difference was observed at 30 min postmortem, lower G6P ($P = 0.0004$) was detected at 120 min in muscles from the RT treatment compared to control. When glycogen is not a limiting factor, G6P accumulates in muscle to reach a concentration of about 10 μmol/g by 24 h postmortem across most meat species (Copenhafer et al., 2006; Apaoblaza et al., 2015; Matarneh et al., 2015). Surprisingly, at 1,440 min, G6P was very low regardless of the chilling treatment,

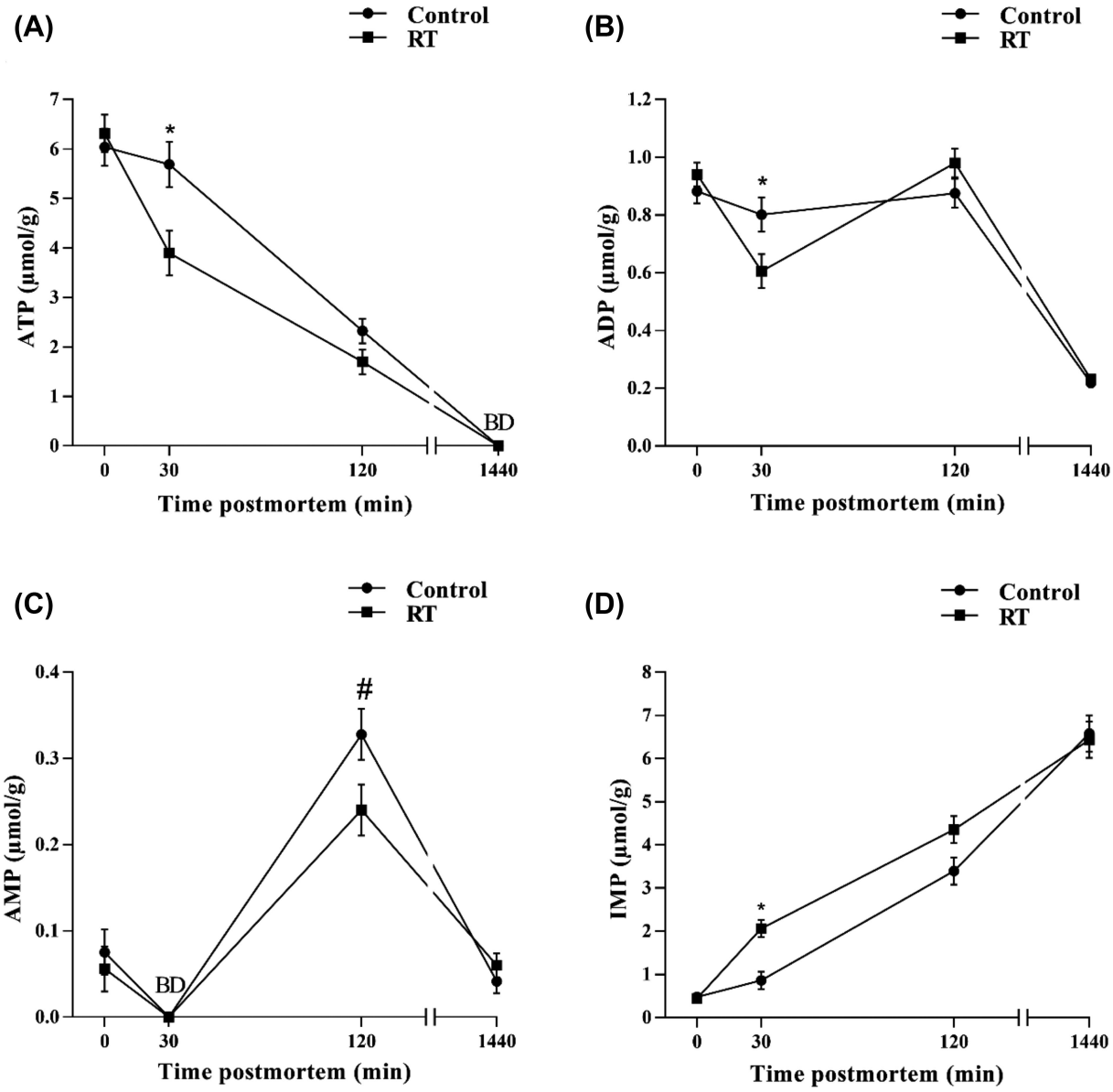

Figure 5. Mean ATP (A; μmol/g), ADP (B, μmol/g), AMP (C; μmol/g) and IMP (D; μmol/g) in broiler P. major muscle of control and RT treatments. Data are LS means ± SE. *indicates significant difference within a time point ($P < 0.05$). #indicates tendency for difference within a time point ($P < 0.1$).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

suggesting that glycogen degradation was either greatly repressed or G6P was generated at a rate comparable to that of consumption. Glucose concentration was also significantly affected by the chilling treatment over time (treatment × time, $P = 0.0002$; Figure 4D). Breast muscles left at room temperature had lower glucose levels at 120 and 1,440 min ($P \leq 0.002$) compared to those from the control treatment. Similar to G6P, glucose concentration in both treatments was lower than that usually observed in postmortem muscle (Apaoblaza et al., 2015; Matarneh et al., 2015). This is likely due to the conversion of glucose to G6P by hexokinase. When G6P levels are high, hexokinase is inhibited through negative feedback mechanisms. On the contrary, however, low G6P concentration, similar to that observed in the current study, removes the inhibition on hexokinase and promote the conversion of glycose to G6P. Correspondingly, the lower glucose content in the RT treatment at 120 and 1,440 min suggests that more glucose was converted to G6P to meet the greater demand.

The rate of postmortem glycolysis is directly related to the rate of ATP hydrolysis (Scopes, 1974). Mean muscle ATP concentration of the RT treatment was lower than control at 30 min postmortem ($P = 0.02$; Figure 5A). At 120 min, no difference was found among treatments, while at 1,440 min ATP was below the limits of detection in both treatments. ATP splitting rate by muscle ATPases decreases with decreasing temperature from 38 to 15°C (Newbold and Scopes, 1967), which explains the greater ATP concentration at 30 min in control treatment. At 30 min postmortem, P. major muscles from the RT treatment had significantly lower

ADP content ($P = 0.03$; Figure 5B) when compared to control. At 120 and 1,440 min, there were no significant differences in ADP among treatments. AMP was below the limits of detection at 30 min postmortem. However, a trend toward greater AMP values were observed in breast muscles from the control treatment at 120 min in comparison to those of the RT ($P = 0.08$, Figure 5C). Greater IMP concentration ($P = 0.04$; Figure 5D) was detected at 30 min in muscles from the RT treatment than control. However, differences in IMP among treatments was not observed at 120 and 1,440 min. As ADP concentration rises due to ATP hydrolysis in postmortem muscle, adenylate kinase catalyzes the dismutation reaction between 2 molecules of ADP to generate ATP and AMP. Once formed, AMP is converted to IMP by AMP deaminase, which leads eventually to the depletion of the adenine nucleotide pool (Greaser, 1986). The loss of adenine nucleotides has previously been shown to mediate premature termination of postmortem metabolism (England et al., 2016). Yet, adenine nucleotides were not depleted by 120 min, suggesting that adenine nucleotides were not limiting, at least during the first 120 min postmortem. Combined with pH and metabolite data, our findings suggest that breast muscle chilling only partially explain the high ultimate pH in broiler P. major muscle. Further, postmortem glycolysis was terminated in the presence of residual glycogen which eliminates glycogen from being the causative agent. On the other hand, however, the low G6P concentration at 1,440 min raises the possibility that glycogenolysis was inhibited, which makes G6P potentially responsible for the termination of postmortem glycolysis.

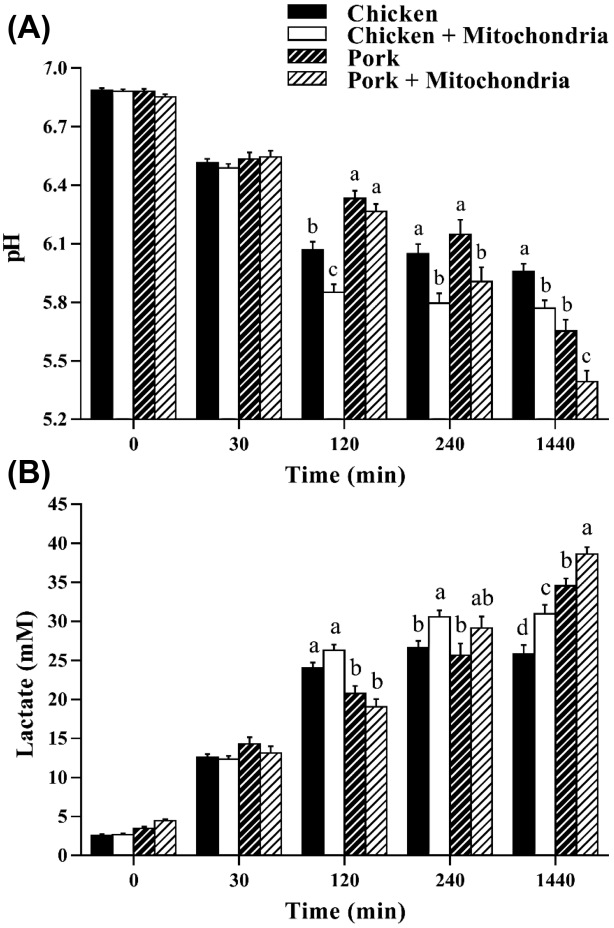

Figure 6. Mean pH (A) and lactate (B; mM) of the in vitro model. Data are LS means ± SE. a, b, c, d means lacking a common letter differ within a time point ($P < 0.05$).

In Vitro pH and Metabolite

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

The pH of the in vitro system was significantly affected by the interaction between species and time ($P < 0.0001$) and mitochondria and time ($P < 0.0001$) (Figure 6A). At 120 min, mitochondria significantly lowered the pH in system containing P. major muscle ($P = 0.0001$), and in both muscles at 240 and 1,440 min ($P < 0.0001$) compared to those containing the same muscle without mitochondria. Reaction vessels containing P. major regardless of mitochondria had lower pH at 120 min ($P < 0.0001$) in comparison to their LL counterparts. At 1,440 min, reaction containing LL muscle with mitochondria had the lowest ($P < 0.0001$) pH value (5.39 ± 0.06), while reactions containing P. major without mitochondria had the highest value (5.96 ± 0.04). Further, no difference in ultimate pH between LL without mitochondria and P. major with mitochondria was detected at 1,440 min. At 120 min, samples containing P. major muscle regardless of mitochondria had greater lactate levels compared to those containing LL muscle ($P = 0.0008$; Figure 6B). At 240, mitochondria contributed to greater lactate accumulation in system containing P. major but not in the ones containing LL muscle ($P = 0.001$). Reactions containing LL muscle with mitochondria had the greatest lactate concentration at 1,440 min, while P. major without mitochondria had the lowest ($P < 0.0001$). Regardless of mitochondria, enhanced glycogen degradation was

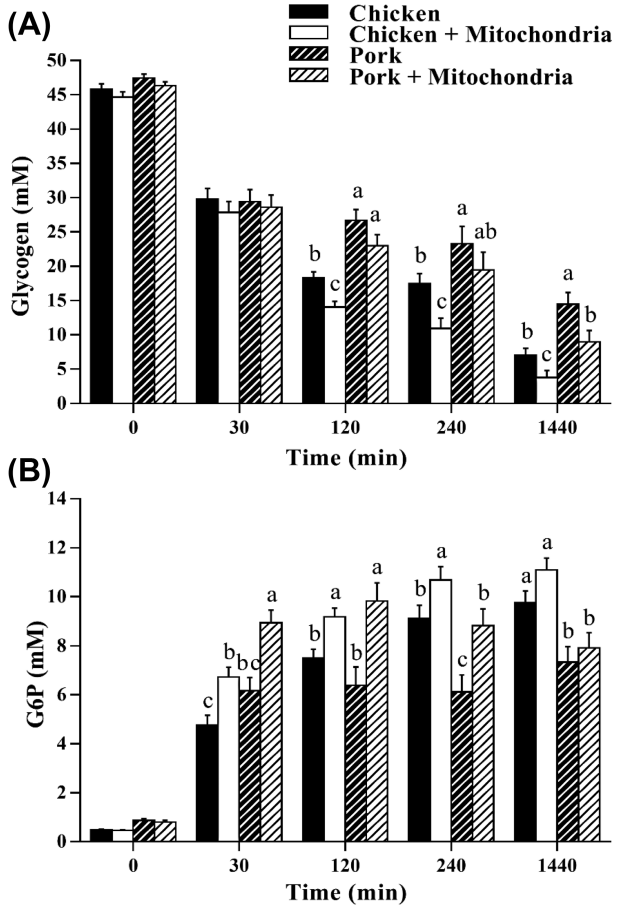

Figure 7. Mean glycogen (A; mM) and G6P (B; mM) of the in vitro model. Data are LS means ± SE. a, b, c means lacking a common letter differ within a time point ($P < 0.05$).

observed in system containing P. major muscle compared to those containing LL muscle from 120 to 1,440 min ($P \leq 0.04$; Figure 7A). Within the same species, mitochondria lowered glycogen concentration in P. major samples at 120 and 240 min and in samples from both species at 1,440 min ($P \leq 0.02$). Samples containing P. major muscle with mitochondria had the lowest glycogen levels from 120 to 1,440 min, while samples containing LL without mitochondria had the greatest levels at the same time points ($P \leq 0.02$). Reaction vessels containing LL with mitochondria had the greatest G6P concentration at 30 min, while the lowest concentration was observed in P. major without mitochondria samples ($P < 0.0001$; Figure 7B). In the presence of mitochondria, greater G6P concentration was detected at 120 min in both muscles ($P < 0.0001$). At 240 and 1,440 min, P. major muscle regardless of mitochondria had greater G6P than those containing LL muscle ($P \leq 0.0002$).

Our in vitro data showed that the pH decline of samples containing P. major muscle without mitochondria arrested prematurely, resulting in a high ultimate pH which was comparable to those measured in vitro (Figure 3). In contrast, the ultimate pH of system containing porcine LL muscle was about 0.3 pH units lower than that of P. major treatment (Figure 6A). The lower ultimate pH in LL muscle treatment was associated with greater flux through the glycolytic pathway, as evidenced by greater lactate accumulation at 1,440 min (Figure 6B). Because muscle samples from both species were homogenized in the exact same buffer, differences in ultimate pH should be a function of the incorporated muscle tissue. It has been previously reported that buffering capacity of broiler breast muscle is greater than pork longissimus muscle (Puolanne and Kivikari, 2000). However, this may not be the case for the current study as P. major and LL muscles were incorporated at 1:10 ratio, thereby making the effect of muscle source to the buffering capacity of the in vitro system negligible.

Contrary to in vitro findings, G6P accumulated in samples containing P. major muscle to reach about 10 mM after 1,440 min, (Figure 7B) which argues against G6P being the reason for the cessation of postmortem metabolism in P. major muscle. The termination of postmortem metabolism in the presences of residual glycogen and glycolytic metabolites is a function of adenine nucleotides depletion or pH-mediated inactivation of PFK (Kastenschmidt et al., 1968; Greaser, 1986; England et al., 2014, 2016). To test whether adenine nucleotides disappearance is the reason for the cessation of postmortem metabolism in P. major muscle, we added 24 h P. major muscle to the in vitro system with 3 mM ATP (pH 5.9). We postulated that if adenine nucleotides were limiting, the addition of ATP would drive additional pH decline. Yet, further pH decline or lactate accumulation was not observed (data not shown), suggesting that adenine nucleotides were not limiting. Correspondingly, these data indicate that pH inactivation of PFK is likely the culprit, a notion supported by the accumulation of G6P in the in vitro system. Once inactivated, flux through PFK is halted leading to the accumulation of G6P and fructose-6-phosphate in postmortem muscle.

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

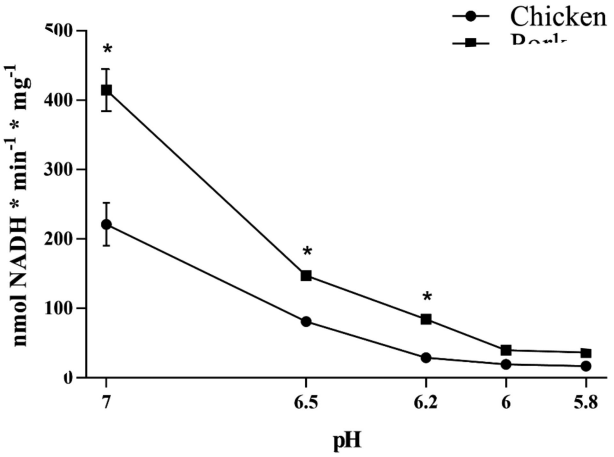

Figure 8. Phosphofructokinase activity comparison between broiler P. major and porcine LL muscle at pH 7, 6.5, 6.2, 6, and 5.8. Data are LS means ± SE. *indicates significant difference within a pH value ($P < 0.05$).

mitochondria can participate to postmortem metabolism and play a role in controlling ultimate pH. Furthermore, variations in the extent of postmortem pH decline may be more thoroughly explained and predicted by the abundance of mitochondria.

PFK Activity

In an attempt to further establish the role that PFK plays in terminating pH decline in broiler P. major muscle, PFK activity was compared between P. major and porcine LL muscle at pH 7, 6.5, 6.2, 6, and 5.8. PFK activity was significantly affected by the interaction between species and pH ($P < 0.0001$; Figure 8). Enzyme activity was dramatically affected by the drop in pH ($P < 0.0001$). Our results show that PFK loses as much as 65% of its activity as pH drops from 7 to 6.5. On the other hand, enzyme activity was about 100% greater in LL than P. major muscle at pH 7 and the difference was maintained at pH 6.5 and 6.2 ($P \leq 0.009$). Yet, the difference was lost at pH 6.0 and 5.8. These findings are consistent with those of (England et al., 2014) who found that PFK activity was markedly impaired due to postmortem pH decline. High concentrations of H^+ promote the dissociation of the more active tetramer form into less active dimer form of the enzyme. Low PFK activity may limit flux through glycolysis leading to premature termination of postmortem metabolism.

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

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