POSTMORTEM REGULATION OF GLYCOLYSIS BY 6-PHOSPHOFRUCTOKINASE IN BOVINE MUSCLE

A Thesis

by

RYAN DOUGLAS RHOADES

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2004

Major Subject: Animal Science

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Approved as to style and content by:

Stephen B. Smith (Chair of Committee)	Jimmy T. Keeton (Member)
Alan R. Sams (Member)	John W. McNeill (Head of Department)

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ABSTRACT

Postmortem Regulation of Glycolysis by 6-Phosphofructokinase in Bovine Muscle.

(August 2004)

Ryan Douglas Rhoades, B.S., Oklahoma State University

Chair of Advisory Committee: Dr. Stephen B. Smith

This study was conducted to assess the regulation of glycolysis by 6-phosphofructokinase (PFK) during the postmortem metabolism of beef muscle. In the first experiment, *M. sternocephalicus* pars mandibularis samples were excised from six randomly-selected steers. Two samples were obtained from each steer immediately postmortem; one sample was quickly immersed in liquid nitrogen and the other was stored at 4^{o}C for 4 d. Glycogen concentrations decreased 45% from d 0 to d 4, and 39.6 μmol/g of glycogen was still present in the tissue at d 4. Concentrations of free glucose increased ($P < 0.001$) from 0.84 μmol/g at d 0 to 6.54 μmol/g at d 4. Fructose-6-phosphate (F6P) and glucose-6-phosphate (G6P) increased ($P < 0.001$) from d 0 to d 4 (2.8-fold and 4.7-fold, respectively). Lactate began accumulating immediately (3.33 μmol/g) and was elevated to 45.9 μmol/g by d 4. Glycolytic potential was 34.4 μmol/g higher ($P < 0.05$) when measured at d 0 than at d 4. The greatest activity of PFK was measured in fresh muscle extracts, between pH 7.4-7.8; by reducing the pH to 7.0, PFK activity was depressed by nearly 50% at 1 mM F6P.

In a second experiment, *M. longissimus* lumborum samples were excised at the 13th thoracic rib location from six randomly-selected steers. Samples were obtained at intervals ranging from 40 min to 24 h postmortem. Glycogen concentrations decreased 45% between 40 and 100 min, and tended ($P \leq 0.10$) to decrease between 100 min and 24 h (from 47 to 32 μmol/g). Concentrations of free glucose increased ($P \leq 0.009$) from 1.0 μmol/g at 40 min to 5.0 μmol/g at 24 h. Concentrations of F6P and G6P increased dramatically after 100 min (muscle pH ≤ 6.5), whereas glycogen depletion appeared to halt by 100 min. Lactate began accumulating almost immediately and tripled in concentration by 24 h. The elevation of G6P and F6P, coupled with the pH sensitivity of PFK, indicate that the postmortem decline in pH ultimately inactivates PFK prior to glycogen depletion.

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It is certainly impossible to give everyone the credit they deserve, and as I sit here and think of all the instrumental people in my life, I am very blessed. First and foremost, I would like to thank my family for your continuous love and support. I firmly believe that developing a person's character comes directly from observing your parents reaction to success and failure. As a result, I want to simply thank my parents for being an exceptional example of how hard work, being courteous to others, and striving for excellence really does pay off. It is obvious that without your support, my aspirations in life are not achievable. To my sisters, you are a very important part of my life, I look up to you both for all of your accomplishments and you have inspired me to do things to the best of my ability. I love you with all of my heart.

I would be negligent, if I did not thank Dr. Stephen B. Smith next. Dr. Smith, thank you for giving me the opportunity to gain the knowledge base and experience necessary to accomplish my goals. Everyday, I am reminded of how fortunate I am to be working with the best. With the utmost respect, I thank you for leading me in the direction toward the career I have always desired. On every level, you are the best influence a student could ask for. I am forever in your debt and certainly owe my entire future to your teaching, ethics, and sincerity. I am grateful knowing that you will be a part of my next educational goal.

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Rebecca Lee McDonald, you are the love of my life and I consider myself truly blessed everyday that you are a part of my life. You are everything I have ever wanted and all I will ever need. In any relationship someone has to sacrifice. Your strength, love, and support is vital to me. As we continue on our journey together I want you to know that everything I do is for you and our future. Thank you for being my best friend and making my goals worth achieving. Finally, I want to thank God, without Him none of this is possible.

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CHAPTER I

INTRODUCTION

In all species of livestock, skeletal muscle is the tissue of major economic importance (Hocquette et al. 1998). Additionally, the metabolism of a muscle tissue postmortem is of primary interest, because ultimate pH plays a vital role in overall meat quality (Scopes, 1974). During postmortem metabolism, muscle converts glycogen into lactic acid via the glycolytic pathway, this determines ultimate pH. Currently, it is well documented that the initial concentration of stored glycogen is depleted and the resulting lactic acid concentration is the basis for ultimate pH. Thus, at varying levels of initial glycogen, a lower or higher ultimate pH will be achieved accordingly. Conversely, little information is available that describes the involvement of 6-phosphofructokinase (PFK) activity on the regulation of postmortem glycolysis. Dalrymple and Hamm (1974) proposed that PFK is a rate limiting enzyme as muscle metabolizes glucose carbon postmortem. Once PFK becomes inactivated, glycogen conversion to lactic acid is halted and ultimate pH is determined. However, little information is available to provide evidence for how and when PFK becomes inactive postmortem.

This thesis follows the style and format of *Meat Science*.

CHAPTER II

REVIEW OF LITERATURE

The depletion of glycogen and accumulation of lactate observed during postmortem metabolism is commonly observed in postmortem muscle (Bendall, 1973; Dalrymple & Hamm, 1974; Lawrie, 1985; Wulf, Emnett, Leheska & Moeller, 2002). These phenomena and the associated decrease in muscle pH are often used to explain variations in meat quality. Specifically, the rate and extent of pH decline has been used to explain differences in muscle color (Apple et al., 1995; Page, Wulf, & Schwotzer, 2001), water holding capacity (Unruh, Kastner, Kropf, Dikeman & Hunt, 1986; McKenna, Maddock & Savell, 2003), and tenderness (Smulders, Marsh, Swartz, Russell & Hoenecke, 1990; Wulf et al., 2002).

Glycolysis is thought to continue in postmortem muscle until substrate stores are depleted or the enzymes which mediate the pathway are inactivated (Lawrie, 1985). Ultimate pH is then established because glycogen can no longer be converted into lactate. During glycolysis, lactate begins to accumulate very rapidly and muscle pH declines. Commensurately, in beef muscle ultimate pH typically is pH 5.5-5.7. However, in the case of dark cutting beef, pH is no lower than pH 6.0-6.2, which is thought to be a result of low initial glycogen concentrations. The measurement of glycolytic potential is commonly used to indicate the amount of glycogen contained in at-death muscle that potentially could be converted to lactate by postmortem metabolism (Hartschuh, Novakofski & McKeith, 2002). Glycolytic potential is calculated as 2 X (glycogen + glucose + glucose-6-phosphate) + lactate (Hartschuh et al., 2002). The

application of glycolytic potential as a predictor of meat quality is based on the assumption that the concentration of glycogen is the primary determinant of ultimate pH. Wulf et al. (2002) related glycolytic potential in the *M. longissimus* lumborum to the incidence of dark cutting (high ultimate pH) beef. These investigators reported that when glycolytic potential will be low (< 80 μmol/g), muscle pH will be high (above 6.0). However, no difference was observed in the ultimate pH of muscles with glycolytic potential ranging from 100 to 180 μmol/g. These results suggest that ultimate pH is determined by glycogen concentrations when these concentrations are low, but not when concentrations are above a certain threshold. This potentially has profound implications for our understanding of the relationship between initial glycogen concentration and ultimate pH.

The bulk of the beef carcasses harvested in the U.S. consistently reach a normal ultimate pH, yet there is considerable variation in initial glycogen concentration. This implies that intermediate regulation beyond glycogen concentration is occurring. Wulf et al. (2002) also reported that when initial glycogen concentration is above a certain threshold, ultimate pH is determined by the pH-induced inhibition of glycolytic enzymes rather than initial glycogen concentration. We hypothesize that PFK is the regulatory glycolytic enzyme.

Glycogen, glucose, glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), and lactate account for as much as 95% of the total glycolytic metabolites at all times in postmortem muscle (Dalrymple & Hamm, 1975). Consequently, specific factors controlling glycolysis can be determined by measuring the concentrations of these

metabolites (Dalrymple & Hamm, 1975). Interestingly, the flux of metabolites during glycolysis is controlled by regulatory enzymes which remain active in postmortem muscle until adenosine triphosphate (ATP) becomes extinct (Hamm, 1977). This suggests that ATP is an important source of energy for enzyme function and that rate of ATP disappearance actually governs the rate of glycolysis (Bendall, 1973).

In at-death muscle, glycogen stores begin to mobilize, providing glucose to the system so that metabolism can continue (Figure 2.1). The initial conversion of glycogen to glucose is catalyzed by glycogen phosphorylase. Glycogen phosphorylase is present in postmortem muscle in an active (GPa) and inactive (GPb) form, and ATP is required in transformation from the inactive to active state. It is noteworthy that, when ATP concentrations decrease, phosphorylase activity increases as muscle works to maintain a continued energy source. Once glycogen glycosyl residues have been phosphorylated, the resulting glucose-1-phosphate is converted to G6P, which becomes committed to the cell for metabolism. Elevated concentrations of G6P at any given time in postmortem muscle have been attributed to increased concentrations of glycogen or decreased concentrations of ATP (Kastenschmidt, Hoekstra & Briskey, 1968). Likewise, concentrations of F6P, which is produced from G6P as glycolysis continues, increase under the same conditions. Collectively, when initial glycogen content is above a certain threshold, products such as G6P and F6P seem to accumulate. PFK is the regulatory enzyme responsible for the conversion of F6P into fructose-1,6-disphosphate ($F16P_2$), and the cell can utilize this hexose-bisphosphate for lactate production. Previous literature suggests that the inhibition of PFK activity may be a possible explanation for

the apparent increase in concentrations of G6P and F6P (Dalrymple & Hamm, 1975; Hamm, 1977). Rosenvold (2001) indicated that the most influential control factor in the glycolytic pathway is PFK. Currently, there is substantial evidence that points toward PFK as a possible control mechanism during postmortem glycolysis, but how or why the enzyme becomes inactive still has not been documented.

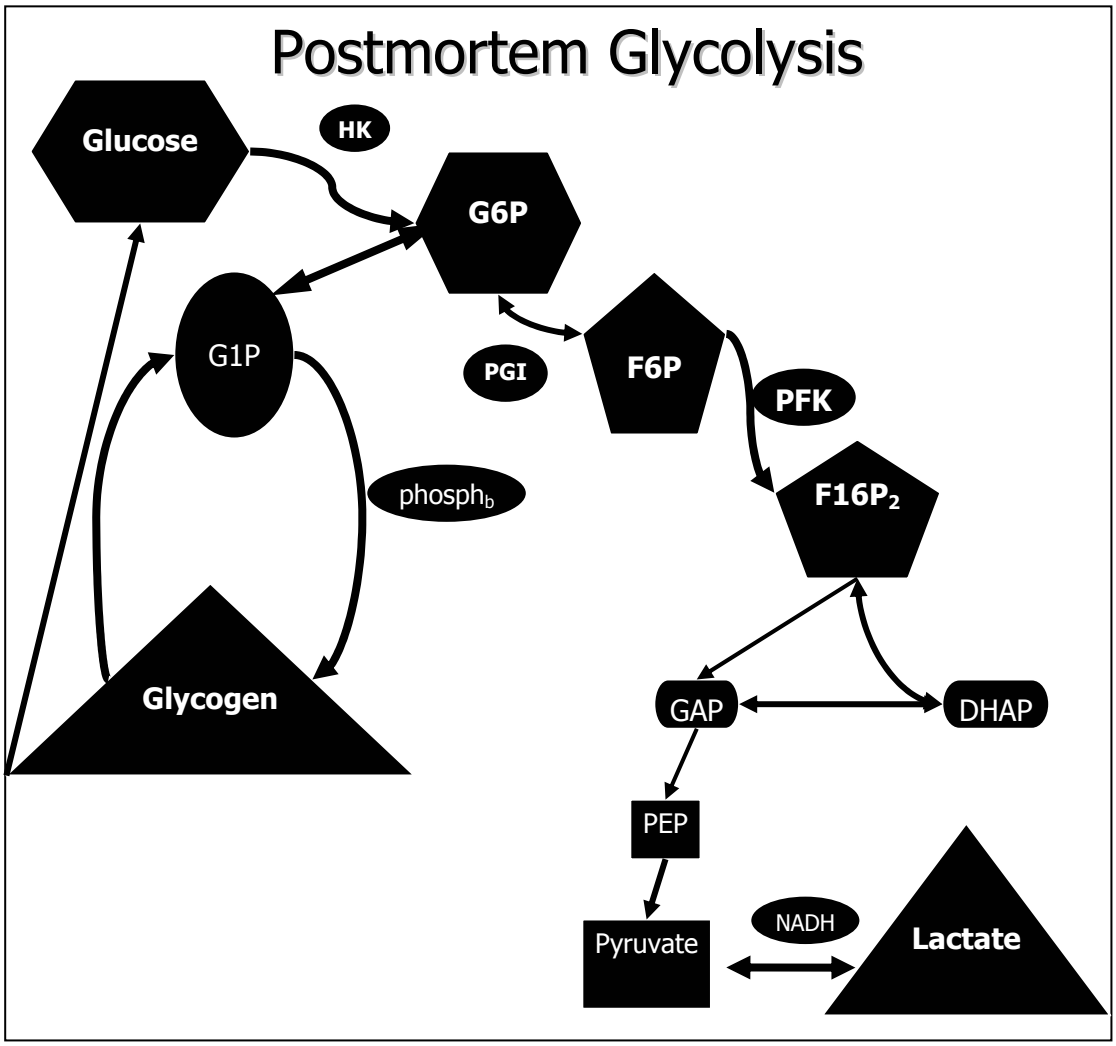

Figure 2.1. Flow chart for glycolytic metabolites during postmortem metabolism.

Trivedi and Danforth (1966) reported that lowering pH reduced the activity of PFK in frog muscle. As glycolysis proceeds, the accumulation of lactate causes an associated decline in pH, which also is responsible for an apparent decrease in the affinity of PFK for substrate (F6P). As a result, substrate levels accumulate, and the increased F6P would allow the flux through PFK. Therefore, in order to indicate a restriction in the flux of glycolysis, preceding metabolites must accumulate. There is novel evidence that the metabolites that precede PFK remain at high and constant levels relative to intermediate concentrations (excluding lactate) subsequent to PFK in beef muscle (Dalrymple & Hamm, 1975). However, the pH sensitivity of PFK and associated inactivation could provide a more precise explanation for how ultimate pH is determined in meat products.

CHAPTER III

POSTMORTEM REGULATION OF GLYCOLYSIS BY 6-PHOSPHOFRUCTOKINASE IN BOVINE *M. STERNOCEPHALICUS* PARS MANDIBULARIS

1. Introduction

In all species of livestock, skeletal muscle is the tissue of major economic importance (Hocquette et al. 1998). Additionally, the metabolism of a muscle tissue postmortem is of primary interest, because ultimate pH plays a vital role in overall meat quality (Scopes, 1974). During postmortem metabolism, muscle converts glycogen into lactic acid via the glycolytic pathway, this determines ultimate pH. Currently, it is well documented that the initial concentration of stored glycogen is depleted and the resulting lactic acid concentration is the basis for ultimate pH. Thus, at varying levels of initial glycogen, a lower or higher ultimate pH will be achieved accordingly. Conversely, little information is available that describes the involvement of 6-phosphofructokinase (PFK) activity on the regulation of postmortem glycolysis. Dalrymple and Hamm (1974) proposed that PFK is a rate limiting enzyme as muscle metabolizes glucose carbon postmortem. Once PFK becomes inactivated, glycogen conversion to lactic acid is halted and ultimate pH is determined. However, there is scant evidence for how and when PFK becomes inactive postmortem. This study is designed to provide novel information about the role that PFK activity plays in determining ultimate pH, by measuring glycolytic intermediate concentrations in postmortem bovine muscle and determining the pH sensitivity of PFK enzyme activity.

2. Materials and methods

2.1. *Sample collection*

Bovine *M. sternocephalicus* pars mandibularis samples were obtained from six randomly-selected steers that would typically be found in a commercial slaughter operation. Cattle were exsanguated at the Rosenthal Meat Science and Technology Center, Texas A&M University, and two samples were excised immediately postmortem from each steer. One sample was then immersed in liquid nitrogen to cease metabolism, whereas the other sample was placed in a cooler at 4^{o}C for 4 d allowing the sample to reach ultimate pH before freezing in liquid nitrogen.

2.2. *Sample preparation*

One gram of frozen muscle was weighed from each sample. To precipitate the protein, 5 ml of 70% perchloric acid was added to the frozen muscle as described by Bergmeyer (1974). The resulting extract was blended using a Polytron homogenizer (Kinematica, Switzerland) at medium setting until all large particles disappeared. The homogenate was stored at -80^{o}C until measurement of glycogen, glucose, G6P, F6P, $F16P_2$, dihydroxyacetone phosphate (DHAP), glyceraldehyde-3-phosphate (GAP), and lactate. Stored homogenate was neutralized (pH 7.0-8.0) by adding 2.5 ml of 1 M $KHCO_3$. The neutralized samples were centrifuged for 15 min at 3,000 xg and the resulting supernate was removed for analysis.

2.3. *Determination of glycolytic intermediate concentrations*

G6P, glucose, and F6P were measured using assay systems described by Bergmeyer (1974). A glycogen buffer system containing 0.9 mM NADP and 1 mM

ATP was added to each cuvette (total volume = 1.0 ml) along with 0.1 ml of extract. Glucose-6-phosphate dehydrogenase (G6PDH) was added to each cuvette catalyzing G6P to 6-phosphogluconate and the change in absorbance was measured using a Beckman DU-7400 Spectrophotometer (Palo Alto, CA) set at 339 nm. Next, glucose was converted to G6P by the addition of hexokinase (HK) and the change in absorbance was measured. Finally, phosphoglucose isomerase (PGI) was added to each cuvette to convert F6P to G6P and the change in absorbance was measured. All measurements of activity were recorded at 0, 5, 10, and 15 min after the enzymes were added to the cuvette.

The same glycogen buffer system (Bergmeyer, 1974) was used to quantify glycogen, with modification. In digesting the homogenate, 0.3 ml of extract was combined with 2.0 ml of amyloglucosidase solution (10 mg/ml). After the samples were placed in a 40^{o}C water bath for 2 h, 2.0 ml of 70% perchloric acid was added to stop the reaction. Then, 0.1 ml of the digested sample was added to each cuvette along with 1.0 ml of ATP/$NADP^{+}$/G6PDH buffer (total volume = 1.0 ml). Background measurements of activity were recorded at 0 and 10 min; then, HK was added to each cuvette and the change in absorbance was read every 5 min, until 15 min after the enzyme was included.

$F16P_2$, DHAP, and GAP were measured using an assay system described by Bergmeyer (1974), with modification. A triethanolamine buffer (0.4 M, pH 7.6) was added (1.5 ml) to each cuvette along with 1.5 ml of extract and 0.1 ml of NADH (8 mg/ml) solution (total volume = 3.1 ml). Baseline activity was recorded at 0 and 5 min, and then a combination of glycerol-3-phosphate dehydrogenase (G3PDH) and

triosephosphate isomerase (TPI) was added to each cuvette and the change in absorbance was measured at 15 min after addition of the coupling enzyme. Aldolase was then added to each cuvette and the change in absorbance was read at 0, 5, 10, and 15 min.

Determination of lactate was as described by Bergmeyer (1974). This procedure was followed without modification and the change in absorbance was read at 90 min.

2.4. *Determination of PFK sensitivity to pH*

A coupled-enzyme assay as described by Bergmeyer (1974) was used to determine the optimum pH for PFK activity, with some modification. One gram of muscle was homogenized in 5 ml of 0.1 M KPO_4 buffer (pH 7.4) and centrifuged for 15 min at 3,000 xg; the supernate was centrifuged again for 30 min at 15,000 xg. Each cuvette contained 1.9 ml Tris buffer (pH 7.0, 7.4, and 7.8), and 0.1 ml 100 mM $MgSO_4$, 0.3 ml 50 mM ATP, 0.1 ml NADH (8 mg/ml), 0.3 ml G3PDH/TPI/aldolase solution, and 0.1 ml of homogenate (total volume = 2.8 ml). Baseline measurements were recorded and then increasing concentrations of F6P were added (0.3 ml), (total volume = 3.1 ml). The change in absorbance was measured at 339 nm.

For all measurements, between 0.5-1.0 U of coupling enzymes was added.

2.5. *Statistical analysis*

Data were analyzed as a randomized complete block using the General Linear Model of the Statistical Analysis System (Version 6.12, SAS Institute, Cary, NC). Animal was used as the blocking factor. The model tested the effect of time postmortem on muscle glycolytic metabolite concentration as the main effect and the PDIFF test contained in the same software was used to separate means when $P < 0.05$.

2.6. Source of chemicals

All chemicals and biochemicals were purchased from Fisher Scientific (Pittsburg, PA, USA) or Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

3. Results

The least squares means for the glycolytic intermediate concentrations in beef *M. sternocephalicus* pars mandibularis immediately after exsanguination or at d 4 postmortem are presented in Table 1. As expected, the concentration of glycogen was highest (86.7 μmol/g) at death and declined as a result of postmortem metabolism. At d 4 postmortem, muscle glycogen concentrations had declined by 45% (to 39.6 μmol/g). In contrast, free glucose, G6P, and F6P had accumulated in muscle by d 4 postmortem to levels that were 7.8-, 2.7-, and 4.75-fold higher, respectively, than at d 0. The muscle concentrations of $F16P_2$ and GAP + DHAP did not differ between postmortem sampling times. The concentration of lactate was low at death, but had increased ($P < 0.001$) dramatically by d 4 postmortem. Glycolytic potential, which is calculated as an indicator of the total potential for lactate production in muscle (Hartschuh et al., 2002), was 34.4 μmol/g higher ($P < 0.05$) at d 0 than at d 4 (Figure 3.1).

Figure 3.2 presents a cross-over diagram comparing the concentrations of each metabolite measured between the d 0 and d 4 samples. Muscle concentrations of free glucose, G6P, and F6P were higher in the muscle samples taken after d 4 postmortem. A cross-over occurred between F6P and $F16P_2$. This indicated that the rate-limiting step for flow of metabolites through the glycolytic pathway occurred at PFK during postmortem metabolism.

Due to the apparent decrease in flux of substrate through PFK during postmortem metabolism, we hypothesized that the decrease in pH observed in postmortem muscle had an adverse effect on the activity of PFK. To test this hypothesis, the activity of PFK in d 0 muscle was determined with increasing substrate concentrations in a buffer system at pH 7.8, 7.4, and 7.0 (Figure 3.3). The greatest PFK activity was observed in extracts at 7.8. PFK activity in muscle extracts buffered to 7.4 displayed somewhat depressed activity compared to the activity observed at 7.8. However, the activity at this pH, which coincides with the pH of living tissue, remained relatively high. Reducing the pH to 7.0 severely depressed PFK activity, displaying readily measurable activity only at 1.0 mM F6P. Even at this substrate concentration, PFK activity was 50% lower than the activity observed at pH 7.8.

Table 1.

Least squares means for glycolytic metabolites of beef *M. sternocephalicus* pars mandibularis muscles analyzed immediately post-exsanguination or 4 d postmortem.

Metabolite	Day postmortem		SEM	P > F
	0 d	4 d		
Glycogen (μmol/g)	86.77[a]	39.60[b]	9.64	<0.001
Free glucose (μmol/g)	0.84[b]	6.54[a]	0.35	<0.001
G6P (μmol/g)[a]	1.65[b]	4.57[a]	0.47	<0.01
F6P (μmol/g)[b]	0.40[b]	1.90[a]	0.21	<0.01
F1,6P2 (μmol/g)[c]	0.06	0.03	0.03	0.38
GAP + DHAP (μmol/g)[d]	0.46	0.43	0.13	0.83
Lactate (μmol/g)	3.33[b]	45.96[a]	1.09	<0.001
Glycolytic potential (μmol/g)[e]	181.84[a]	147.39[b]	20.08	0.01

[a]G6P = Glucose-6-phosphate.

[b]F6P = Fructose-6-phosphate.

[c]F1,6P2 = Fructose-1,6-bisphosphate.

[d]GAP + DHAP = Glyceraldehyde-3-phosphate + dihydroxyacetone phosphate.

[e]Glycolytic potential = 2 X (glycogen + glucose + G6P) + lactate.

Least squares means lacking common letters ([a,b]) differ ($P < 0.05$)

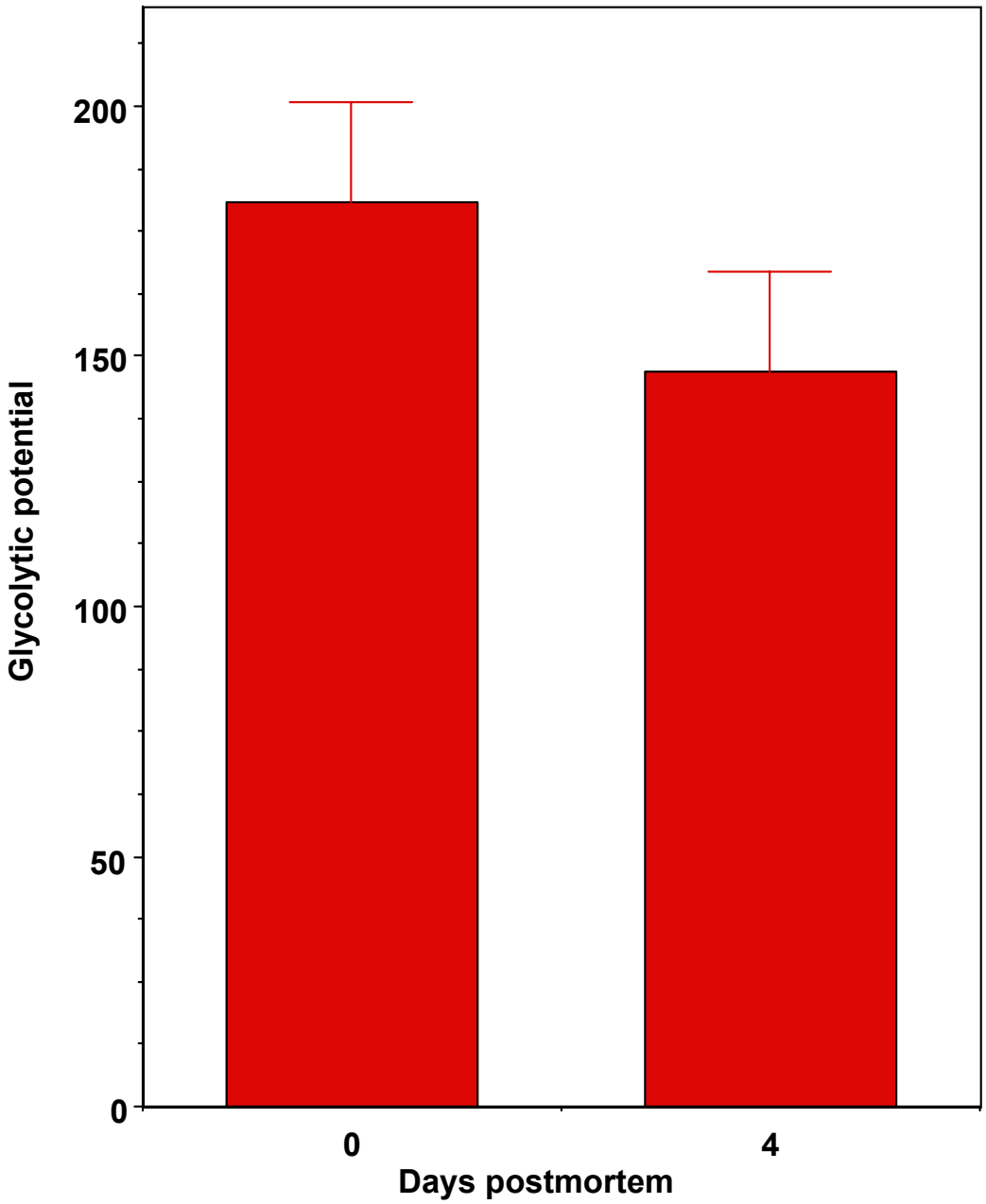

Figure 3.1. Least squares means for glycolytic potential in *M. sternocephalicus* pars mandibularis muscle sampled at d 0 and d 4. Data are representative of six replicates, means ± SEM.

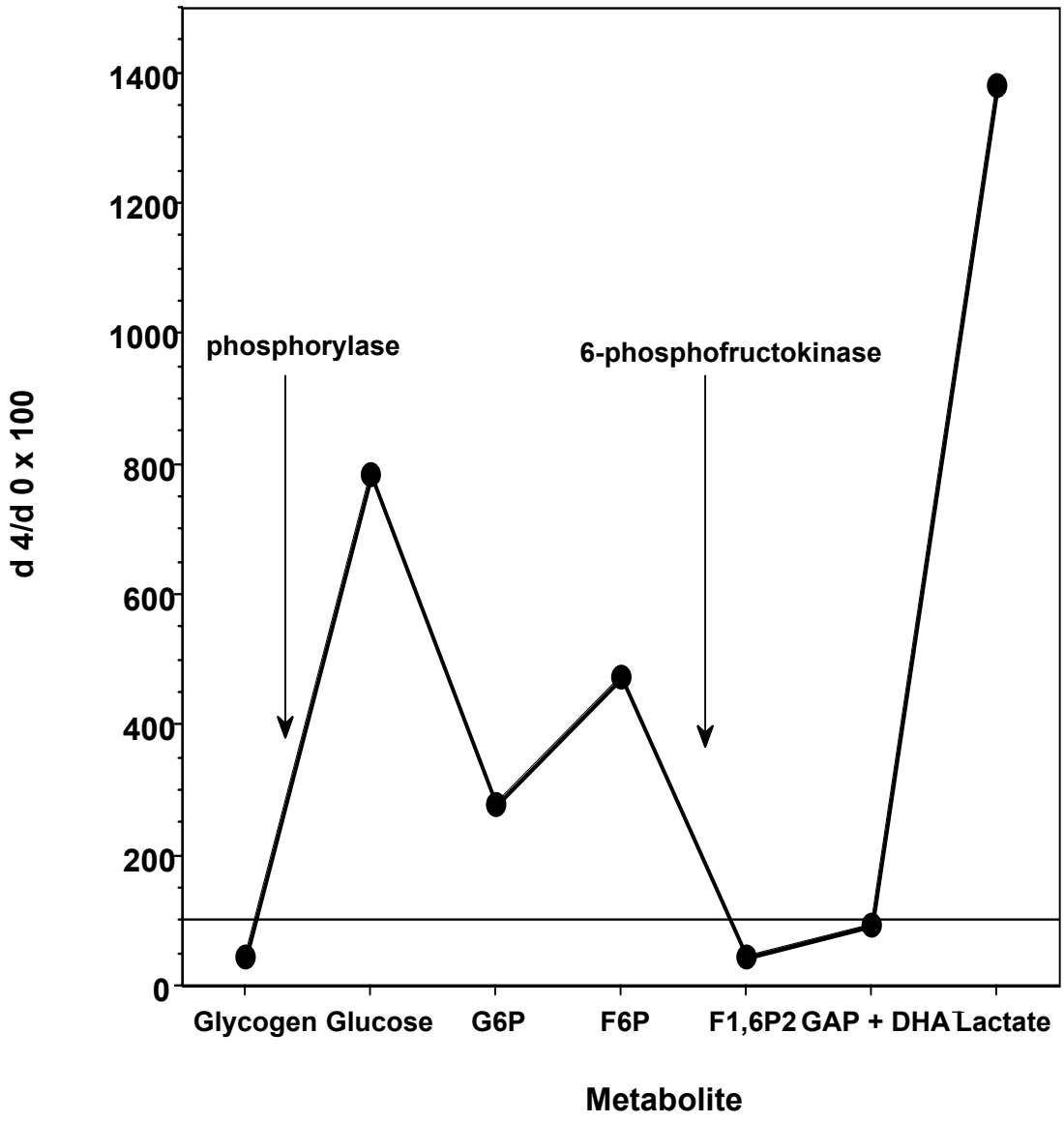

Figure 3.2. Cross-over diagram for glycolytic metabolites of beef *M. sternocephalicus* pars mandibularis muscles sampled at death or at 4 d postmortem. Values are the ratios of (concentrations at d 4):(concentrations at d 0) X 100. Data are means of six replicates.

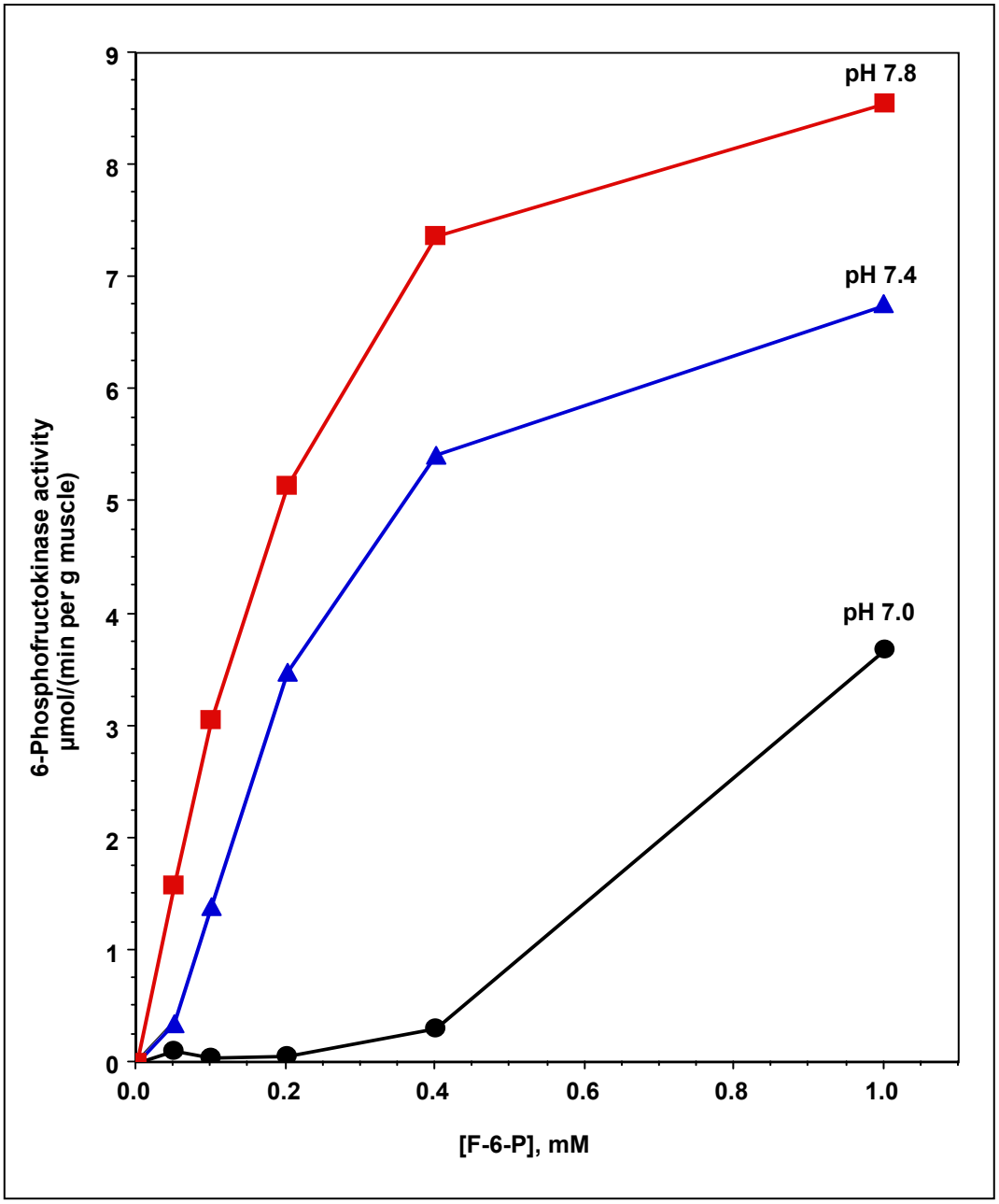

Figure 3.3. Activity of 6-phosphofructokinase at 7.8, 7.4, and 7.0 in beef *M. sternocephalicus* pars mandibularis muscles measured 30 min postmortem. Data are representative of six replicates. F-6-P, fructose-6-phosphate.

4. Discussion

The depletion of glycogen and accumulation of lactate observed during postmortem metabolism is commonly observed in postmortem muscle. These phenomena and the associated decrease in muscle pH are often used to explain variations in meat quality. Specifically, the rate and extent of pH decline has been used to explain differences in muscle color (Apple et al., 1995; Page et al., 2001), water holding capacity (Unruh et al., 1986; McKenna et al., 2003), and tenderness (Smulders et al., 1980; Wulf et al., 2002). Glycolysis is thought to continue in postmortem muscle until substrate stores are depleted or the enzymes which mediate the pathway are inactivated (Lawrie, 1985). Our data provide evidence that inactivation of PFK is responsible for the cessation of glycolysis in beef muscle.

Glycolytic potential is commonly used to indicate the amount of glycogen contained in at-death muscle that potentially could be converted to lactate by postmortem metabolism (Hartschuh et al. 2002). The application of glycolytic potential as a predictor of meat quality is based on the assumption that the concentration of glycogen is the primary determinant of ultimate pH. The apparent decline in glycolytic potential between d 0 and d 4 suggests that some oxidative metabolism of the glucose carbon occurred in the muscle postmortem. This would contribute to the inaccuracy of glycolytic potential as a predictor of meat quality. Wulf et al. (2002) related glycolytic potential in the *M. longissimus lumborum* to the incidence of dark cutting (high ultimate pH) beef. These investigators reported that when glycolytic potential was low (< 80 μmol/g), muscle pH was high (above 6.0). However, no difference was observed in the

ultimate pH of muscles with glycolytic potential ranging from 100 to 180 μmol/g. These results suggest that ultimate pH is determined by glycogen concentrations when these concentrations are low, but not when concentrations are above a certain threshold. This potentially has profound implications for our understanding of the relationship between initial glycogen concentration and ultimate pH.

It is noteworthy that free glucose, G6P, and F6P accumulated in muscle during postmortem storage. The accumulation of free glucose, which arises from glycogen debranching, is consistent with the rapid initial degradation of glycogen early postmortem. The accumulation of G6P and F6P suggests that postmortem glycolysis is regulated at PFK. The accumulation of G6P would inhibit both glycogen phosphorylase$_b$ and hexokinase (Kaneko, Harvey & Bruss, 1997), limiting further influx of glucose carbon into glycolysis. Furthermore, the reduced activity of PFK with lower pH suggests that this regulation is due, in part, to the pH sensitivity of the PFK enzyme. In agreement with the present study, Trivedi and Danforth (1966) reported that lowering pH reduced the activity of PFK in frog muscle. However, these investigators found increased substrate (F6P) would allow the flux of substrate through the enzyme. The pH sensitivity of PFK demonstrated in the present study indicates that at physiological substrate concentrations, PFK will continue to function, but at a dramatically reduced rate. This is a possible explanation for the accumulation of the metabolites preceding PFK in the pathway in the d-4 postmortem samples. Consequently, it would appear that the ultimate muscle pH is determined by substrate concentrations in relation to the pH-mediated inactivation of the PFK enzyme.

5. Conclusions

The profound sensitivity of PFK to a small change in pH strongly indicates that it will be inactivated early postmortem. Any subsequent glycolysis proceeds at a lower rate, only by mass action of the elevated F6P. Further research is needed in an economically important cut of meat.

CHAPTER IV

POSTMORTEM REGULATION OF GLYCOLYSIS BY 6-PHOSPHOFRUCTOKINASE IN BOVINE *M. LONGISSIMUS* LUMBORUM

1. Introduction

Ultimate pH is perhaps the most significant factor when determining the overall quality of a meat product (Scopes, 1974). Therefore, it becomes important to the industry on both an economic and consumer level. For example, from an economic perspective, dark-firm-dry (DFD) beef costs the U.S. industry $165 million annually (McKenna et al. 2002). DFD has an above normal ultimate pH (6.0-6.2), which causes the product to be darker in color with less consumer appeal and poor functionality. The higher than normal ultimate pH is thought to be derived from a below normal muscle glycogen concentration at the time of slaughter (Wulf et al. 2002). Nevertheless, a precise definition of how ultimate pH is achieved might provide the industry with some insight on how to reduce this costly problem. From a consumer viewpoint, the effects of ultimate pH on beef tenderness have also been well documented, showing a strong relationship exists between them (Calkins et al. 1983). Because the decline in postmortem pH influences protein degradation, a product not reaching a normal ultimate pH will be less tender (Hocquette et al. 1998). Tenderness is generally perceived as the most influential factor when determining the consumer acceptability of a meat product (Deatherage & Garnatz, 1952). Hence, it is easily concluded that the factors involved in determining ultimate pH are important to the entire scope of the beef industry. We

hypothesize that the rate of inactivation of PFK determines ultimate pH, not the initial glycogen concentration.

2. Materials and methods

2.1. Sample Collection

All samples were obtained from the Rosenthal Meat Science and Technology Center, Texas A&M University. *M. longissimus* lumborum muscle samples were obtained from six steers that would typically be found in a commercial slaughter operation. Times at which samples were taken were 40, 60, 80, 100, 120 min, 6, and 24 h postmortem. All samples were excised from the 13th thoracic rib location. As the samples were taken, muscle pH was measured using a pH meter 440 (Corning Inc., Corning, NY) and the samples were immediately placed in liquid nitrogen. The loin samples were stored at -80^{o}C until further analyses.

2.2. Sample Preparation

One gram of frozen muscle was weighed from each sample. To precipitate the protein, 5 ml of 70% perchloric acid was added to the frozen muscle as described by Bergmeyer (1974). The resulting extract was blended using a Polytron homogenizer (Kinematica, Switzerland) at medium setting until all large particles disappeared. The homogenate was stored at -80^{o}C until measurement of glycogen, glucose, G6P, F6P, $F16P_2$, DHAP, GAP, and lactate. Stored homogenate was neutralized (pH 7.0-8.0) by adding 2.5 ml of 1 M $KHCO_3$. The neutralized samples were centrifuged for 15 min at 3,000 xg and the resulting supernate was removed for analyses.

2.3. Determination of glycolytic intermediate concentrations

G6P, glucose, and F6P were measured using assay systems described by Bergmeyer (1974). A glycogen buffer system containing 0.9 mM NADP and 1 mM ATP was added to each cuvette (total volume = 1.0 ml) along with 0.1 ml of extract. Glucose-6-phosphate dehydrogenase (G6PDH) was added to each cuvette catalyzing the conversion of G6P to 6-phosphogluconate and the change in absorbance was measured using a Beckman DU-7400 Spectrophotometer (Palo Alto, CA) set at 339 nm. Next, glucose was converted to G6P by the addition of hexokinase (HK) and the change in absorbance measured. Finally, phosphoglucose isomerase (PGI) was added to each cuvette to convert F6P to G6P and the change in absorbance was measured. All measurements of activity were recorded at 0, 5, 10, and 15 min after the enzymes were added to the cuvette.

The same glycogen buffer system (Bergmeyer, 1974) was used to quantify glycogen, with modification. In digesting the homogenate, 0.3 ml of extract was combined with 2.0 ml of amyloglucosidase solution (10 mg/ml). After the samples were placed in a 40^{o}C water bath for 2 h, 2.0 ml of 70% perchloric acid was added to stop the reaction. Then, 0.1 ml of the digested sample was added to each cuvette along with 1.0 ml of ATP/$NADP^{+}$/G6PDH buffer (total volume = 1.0 ml). Background measurements of activity were recorded at 0 and 10 min; then, HK was added to each cuvette and the change in absorbance was read every 5 min, until 15 min after the addition of HK.

$F16P_2$, DHAP, and GAP were measured using an assay system described by Bergmeyer (1974), with modification. A triethanolamine buffer (0.4 M, pH 7.6) was

added (1.5 ml) to each cuvette along with 1.5 ml of extract and 0.1 ml of NADH (8 mg/ml) solution (total volume = 3.1 ml). Baseline activity was recorded at 0 and 5 min, and then a combination of glycerol-3-phosphate dehydrogenase (G3PDH) and triosephosphate isomerase (TPI) were added to each cuvette and the change in absorbance was measured at 15 min after addition of the coupling enzyme. Aldolase was then added to each cuvette and the change in absorbance read at 0, 5, 10, and 15 min.

Determination of lactate was as described by Bergmeyer (1974). This procedure was followed without modification and the change in absorbance read at 90 min, as this reaction required more time to reach completion.

2.4. Determination of PFK sensitivity to pH

A coupled-enzyme assay as described by Bergmeyer (1974) was used to determine the optimum pH of PFK activity, with some modification. One gram of muscle is homogenized in 5 ml of 0.1 M KPO_4 buffer (pH 7.4) and centrifuged for 15 min at 3,000 xg; the supernate was centrifuged again for 30 min at 15,000 xg. Each cuvette contained 2.2 ml Tris buffer (pH 7.0, 7.4, 7.8, 8.2, 8.6, and 9.0) and 0.1 ml 100 mM $MgSO_4$, 0.3 ml 50 mM ATP, 0.1 ml NADH (8 mg/ml), 0.1 ml G3PDH/TPI/aldolase solution, and 0.2 ml of homogenate (total volume = 3.0 ml). Baseline activity was recorded at 0 min and then F6P was added to a final concentration of 1 mM. Activity was measured spectrophotometrically at 339 nm.

For all measurements, between 0.5-1.0 U of coupling enzyme was added.

2.5. Statistical analysis

Data were analyzed as a randomized complete block using the General Linear Model of the Statistical Analysis System (Version 6.12, SAS Institute, Cary, NC). Animal was used as the blocking factor. The model tested the effect of time postmortem on muscle glycolytic metabolite concentration as the main effect and the PDIFF test contained in the same software will be used to separate means when $P < 0.05$.

2.6. Source of chemicals

All chemicals and biochemicals were purchased from Fisher Scientific (Pittsburg, PA, USA) or Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

3. Results

The least squares means for the glycolytic intermediate concentrations in beef *M. longissimus* lumborum over time postmortem are presented in the figures that follow. As expected, the concentration of glycogen was highest (87.0 μmol/g) at 40 min and declined as a result of postmortem metabolism. As shown in Figure 4.1, glycogen concentrations decreased 45% between 40 and 100 min, and tended ($P < 0.10$) to decrease between 100 min and 24 h (from 47 to 32 μmol/g). Concentrations of free glucose increased ($P < 0.009$) from 1.0 μmol/g at 40 min to 5.0 μmol/g at 24 h (Figure 4.2). G6P accumulation occurred between 6 and 24 h (Figure 4.3), whereas F6P began to accumulate by 6 h (Figure 4.4). Overall, concentrations of G6P and F6P increased dramatically after 100 min (muscle pH < 6.5), whereas glycogen depletion appeared to halt by 100 min. Lactate began accumulating almost immediately and tripled in concentration by 24 h (Figure 4.5). The accumulation of lactate in postmortem muscle

was directly related to the observed decline in pH values over time (Figure 4.6). Although statistical separation was not achieved, glycolytic potential was numerically higher at 40 min and declined to 120 μmol/g at 24 h (Figure 4.7). These data are consistent with the results found in *M. sternocephalicus* pars mandibularis muscle outlined in Chapter III.

Figure 4.8 presents a cross-over diagram comparing the concentrations of each metabolite measured between the 60 min and 24 h samples. Muscle concentrations of free glucose, G6P, and F6P were higher in the muscle samples taken at 24 h postmortem. A cross-over occurred between F6P and $F16P_2$. This indicated that the rate-limiting step for the flow of metabolites through the glycolytic pathway occurred at PFK during postmortem metabolism.

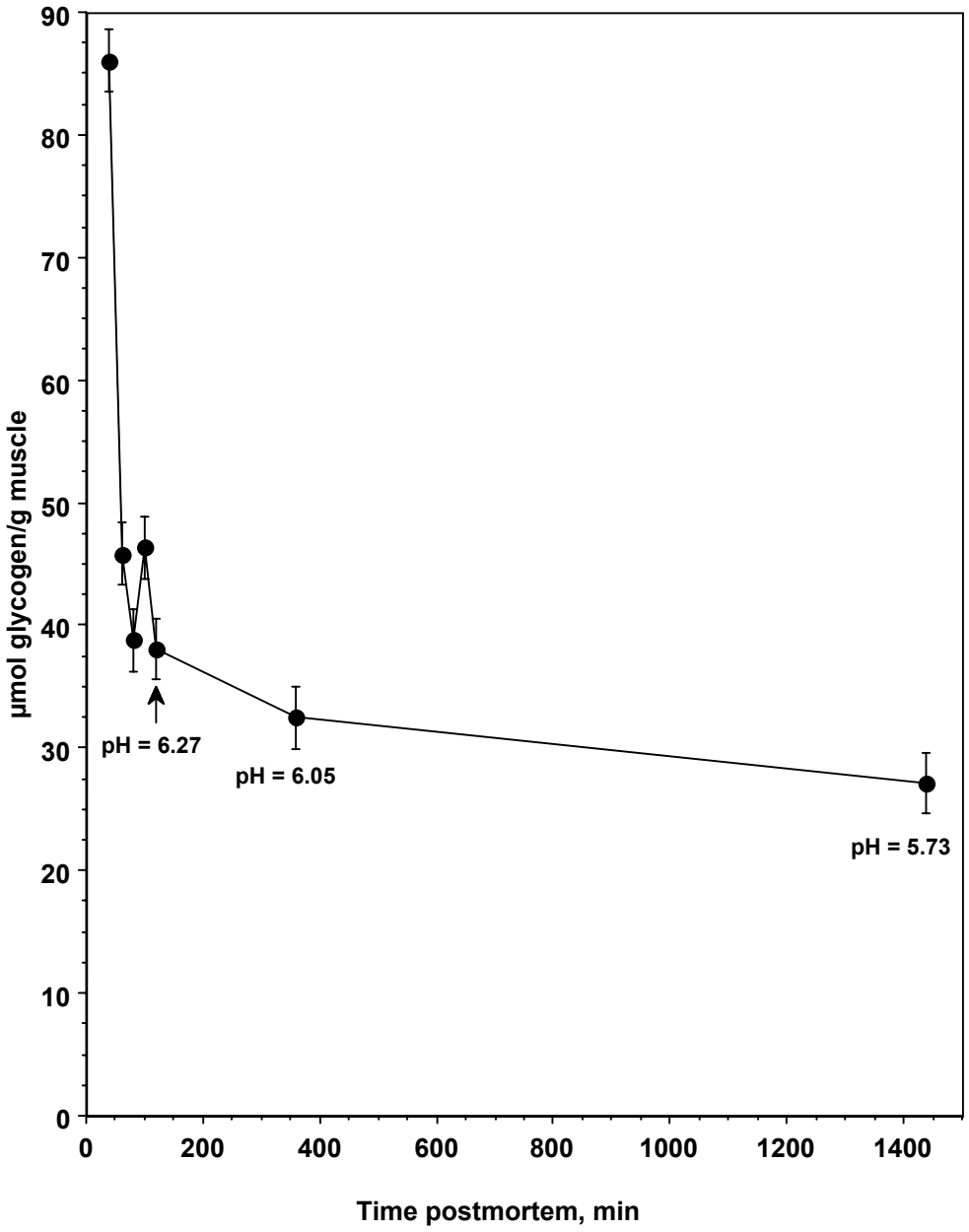

Figure 4.1. Least squares means for glycogen concentrations in *M. longissimus* lumborum muscle sampled over time postmortem. Data are representative of six animals (means ± SEM) except at 40 min postmortem, in which one animal was used.

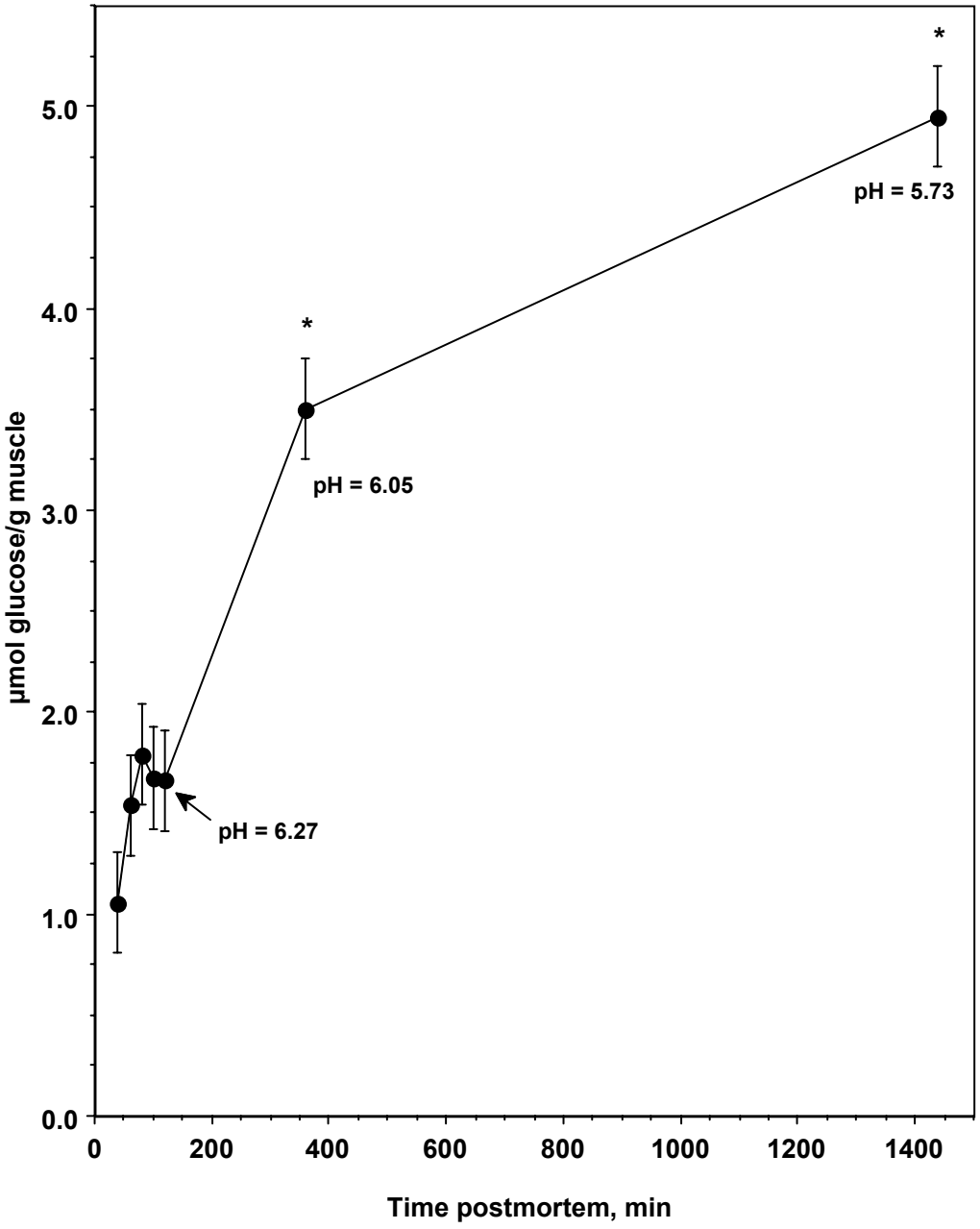

Figure 4.2. Least squares means for free glucose concentrations in *M. longissimus* lumborum muscle sampled over time postmortem. Data are representative of six animals (means ± SEM) except at 40 min postmortem, in which one animal was used.

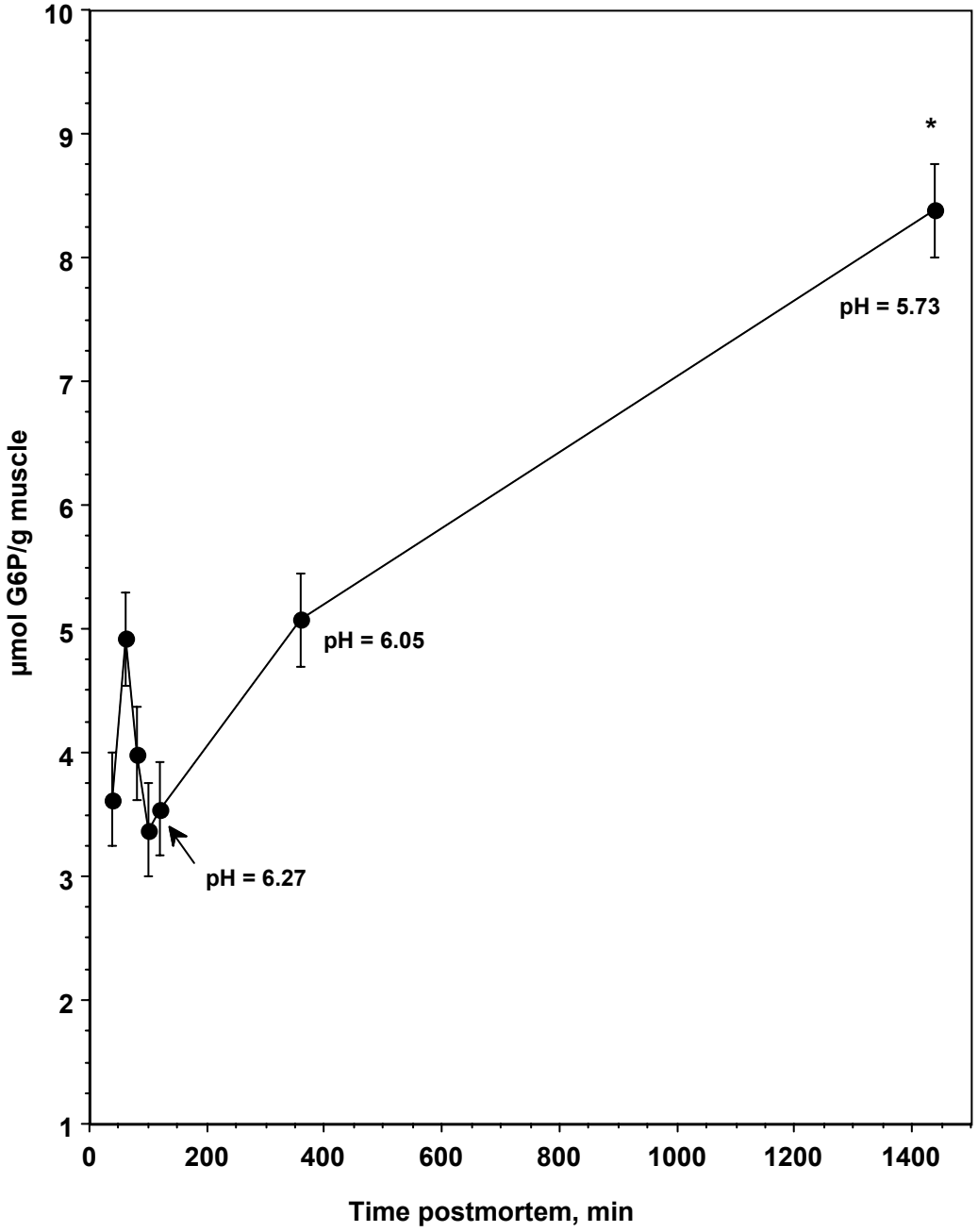

Figure 4.3. Least squares means for G6P concentrations in *M. longissimus* lumborum muscle sampled over time postmortem. Data are representative of six animals (means ± SEM) except at 40 min postmortem, in which one animal was used.

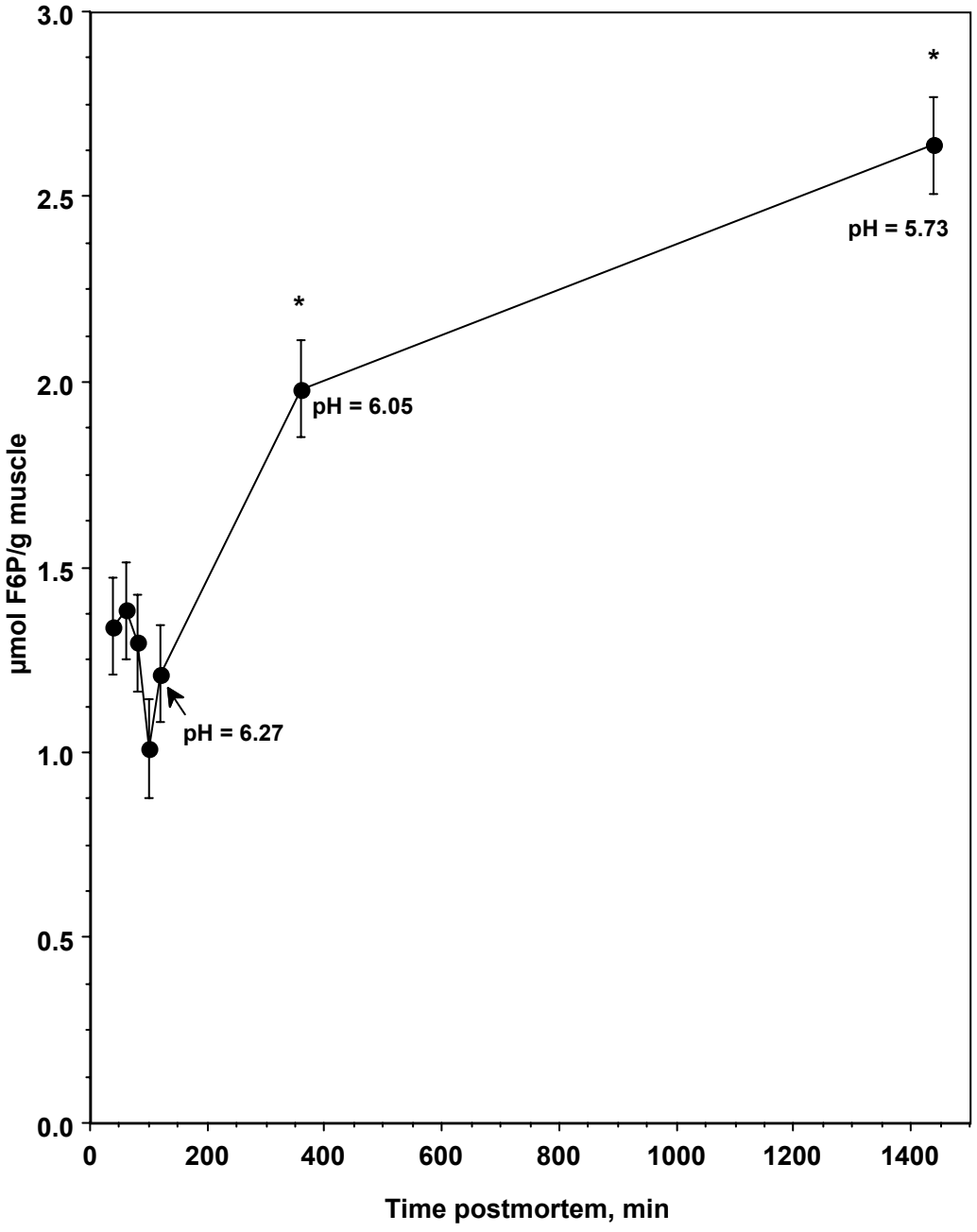

Figure 4.4. Least squares means for F6P concentrations in *M. longissimus* lumborum muscle sampled over time postmortem. Data are representative of six animals (means ± SEM) except at 40 min postmortem, in which one animal was used.

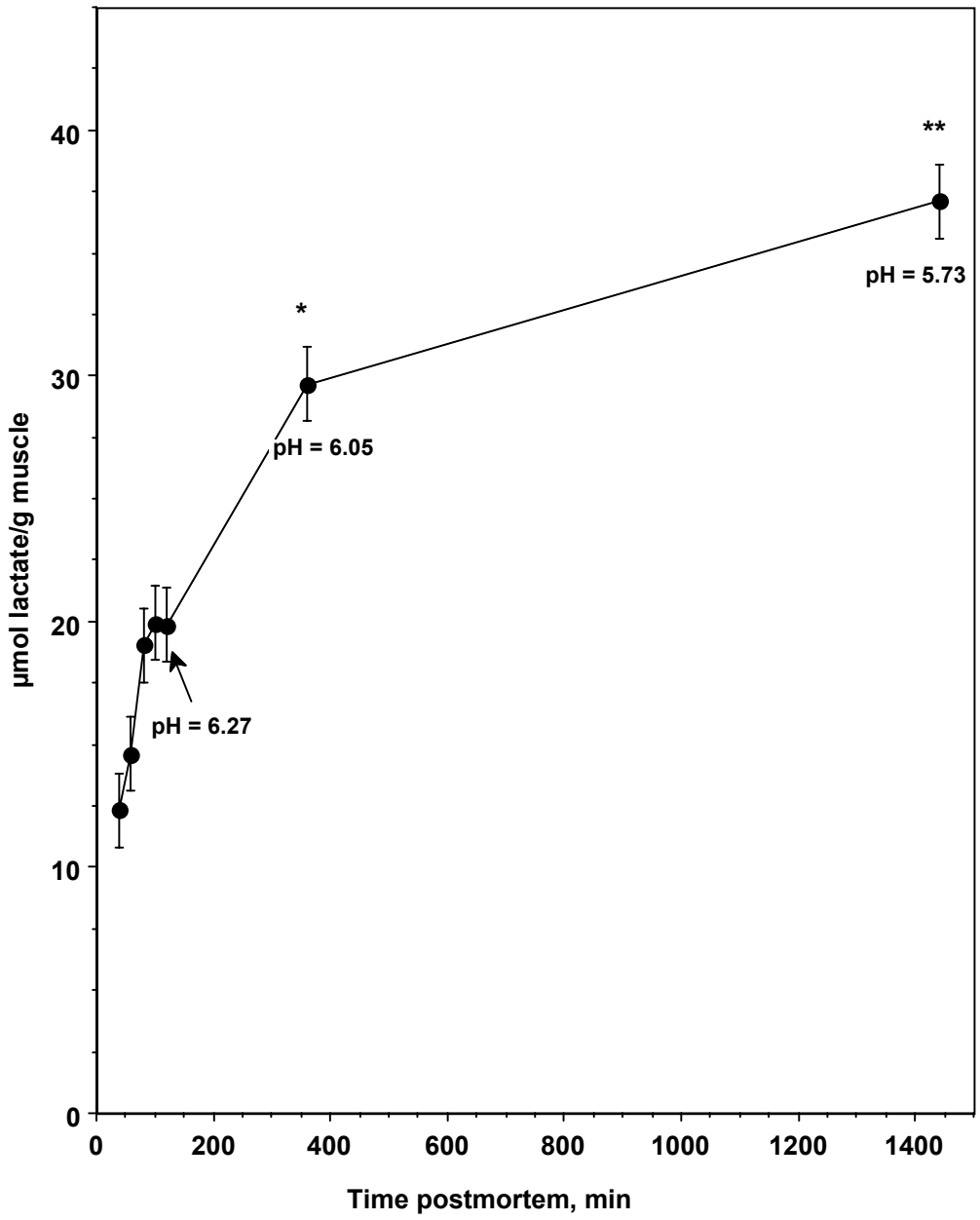

Figure 4.5. Least squares means for lactate concentrations in *M. longissimus* lumborum muscle sampled over time postmortem. Data are representative of six animals (means ± SEM) except at 40 min postmortem, in which one animal was used.

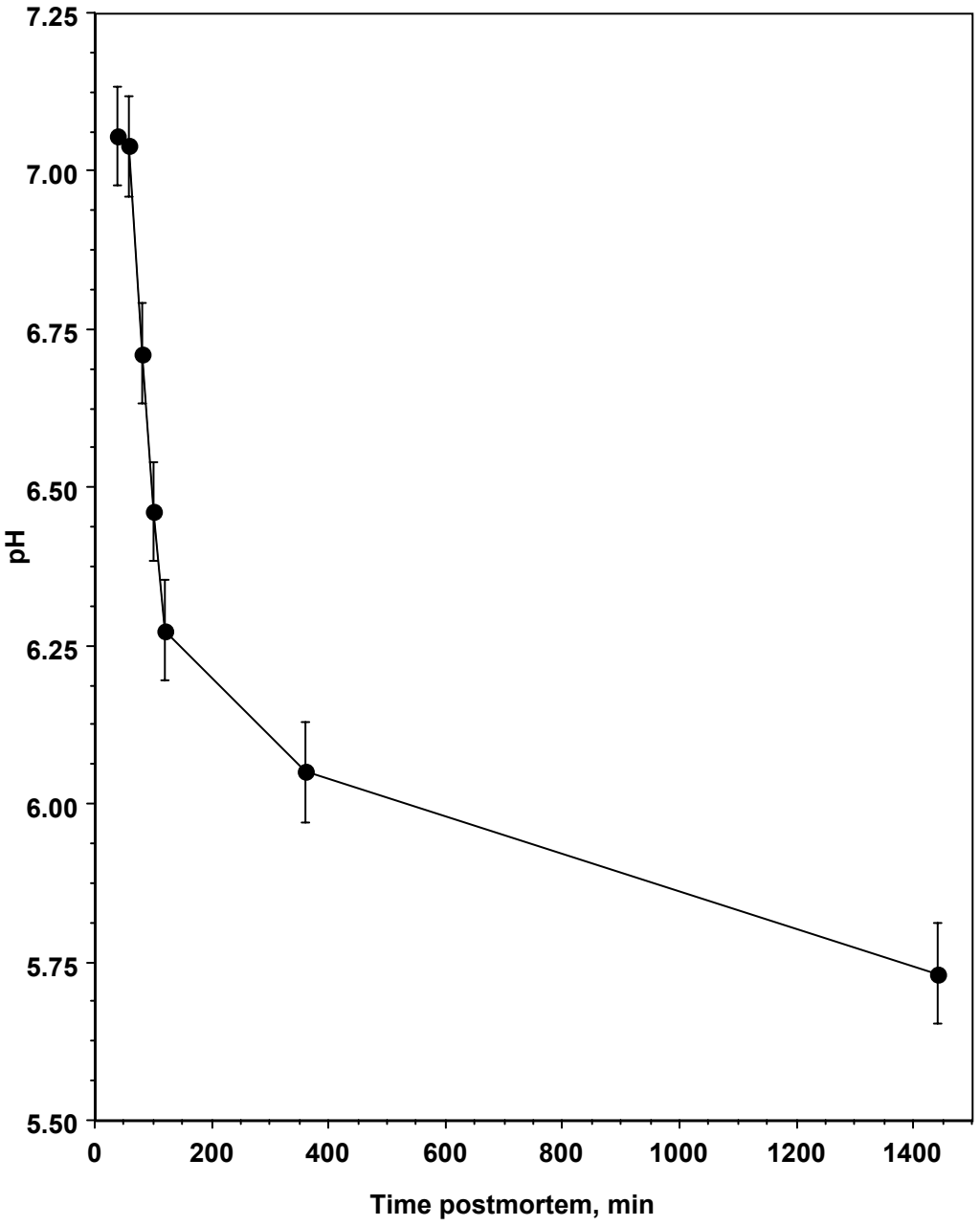

Figure 4.6. Least squares means for pH values in *M. longissimus* lumborum muscle sampled over time postmortem. Data are representative of six animals (means ± SEM) except at 40 min postmortem, in which one animal was used.

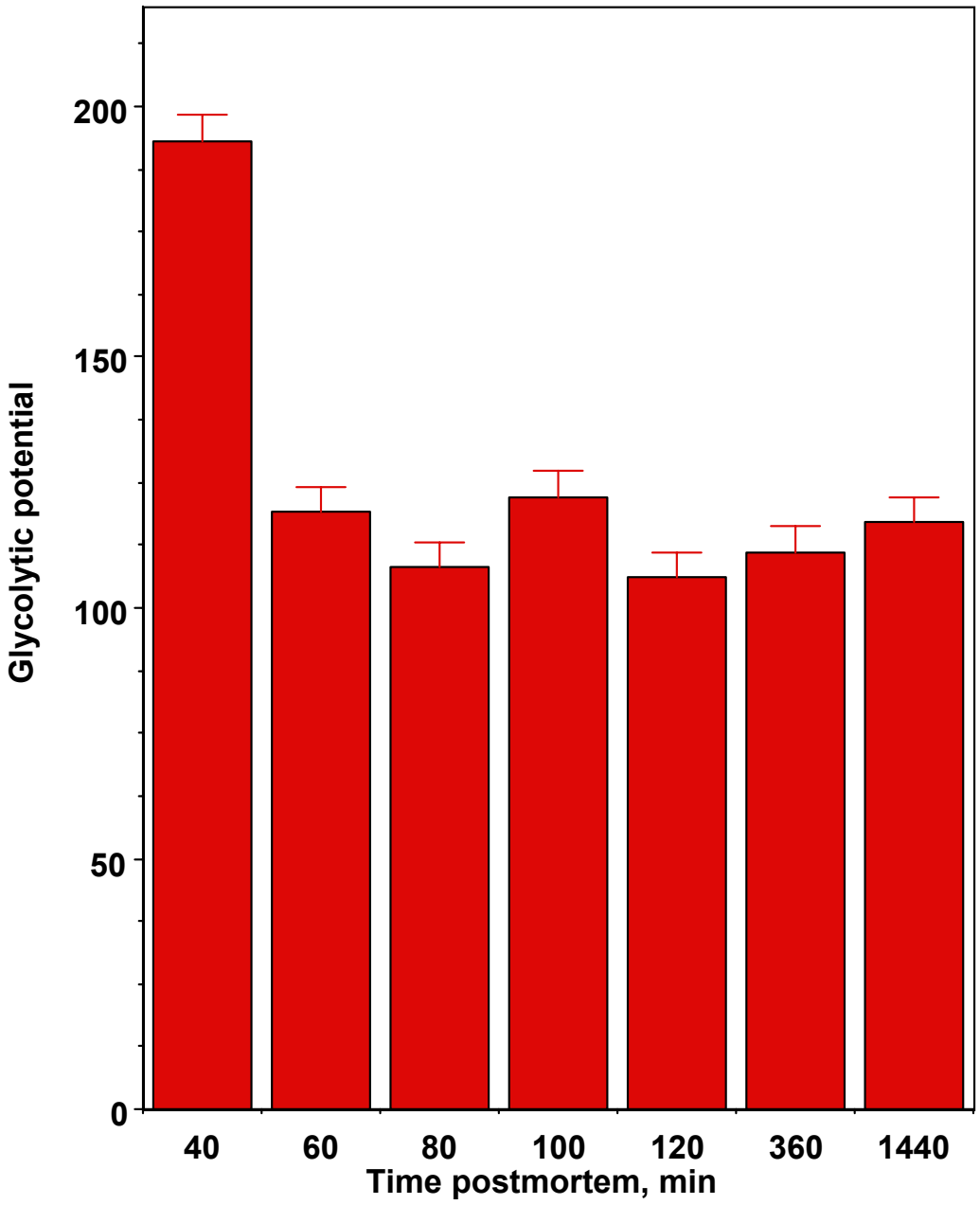

Figure 4.7. Least squares means for glycolytic potential in *M. longissimus* lumborum muscle sampled over time postmortem. Data are representative of six animals (means ± SEM) except at 40 min postmortem, in which one animal was used.

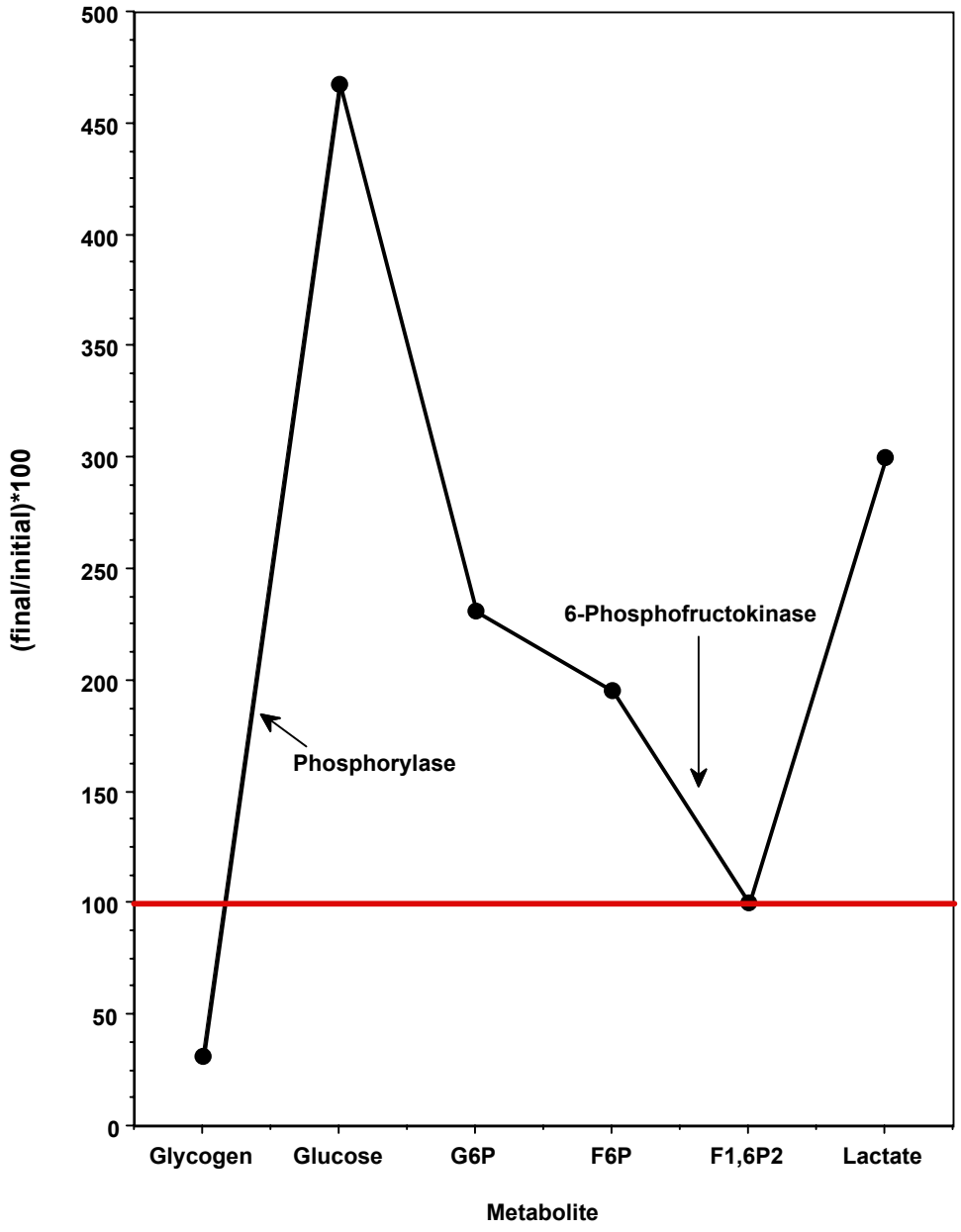

Figure 4.8. Cross-over diagram for glycolytic metabolites of beef *M. longissimus* lumborum muscle sampled over time postmortem. Values are the ratios of (concentrations at 24 h):(concentrations at 60 min) X 100.

4. Discussion

The notion suggesting that the determination of ultimate pH in bovine muscle is derived solely from initial glycogen concentration assumes that stored glycogen is quantitatively converted into lactic acid. The associated accretion of lactate is responsible for the rate and extent of pH decline of postmortem metabolism. This is of significant economic value to the industry because the decline in postmortem pH heavily influences meat quality. In the case of dark cutting beef, in which pH is no lower than pH 6.0-6.2, the higher than normal ultimate pH is thought to be derived from a below normal muscle glycogen concentration at the time of slaughter (Wulf et al. 2002). Although there are studies that support the theory of initial glycogen concentration being the basis for determination of ultimate pH in muscle, our data indicate that the cessation of glycolysis is due to the inactivation of PFK, when glycogen concentration is above a certain threshold. Wulf et al. (2002) also reported that when initial glycogen concentration is above a certain threshold, ultimate pH is determined by the pH-induced inhibition of glycolytic enzymes rather than initial glycogen concentration.

In agreement with the results found in *M. sternocephalicus* pars mandibularis samples, free glucose, G6P, and F6P accumulated in the *M. longissimus* lumborum muscle over time postmortem. In particular, these metabolites increased in concentration dramatically after 100 min postmortem, while the rate of glycogen depletion appeared to diminish at the same time. Previous literature suggests that the inhibition of PFK activity may be a possible explanation for the apparent increase in concentrations of G6P and F6P (Dalrymple & Hamm, 1975; Hamm, 1977). Due to the

observed accumulation of G6P and F6P, this study provides further evidence supporting the idea that glycolysis is regulated by PFK, even in postmortem muscle. In contrast, the apparent increase in free glucose, which is derived from glycogen debranching, is accounted for by a substantial amount of glycogen depletion after the first 100 min. The measurement of glycolytic potential (2 X [glycogen + glucose + G6P] + lactate) is commonly used to predict meat quality, and is based on the theory that total glycogen concentration can be utilized to determine ultimate pH (Hartschuh et al., 2002). In the present study, glycolytic potential declined nearly 70 μmol/g from 40 min to 24 h. This suggests that total glycogen is not utilized by the muscle during postmortem storage and some oxidative metabolism of glucose carbon must have occurred. The fact that the glycolytic potential calculation does not take into account the accumulation of F6P, DHAP/GAP, or pyruvate, could explain the apparent decline in glycolytic potential over time postmortem. To our knowledge, this study represents the first comparison of glycolytic potential across time for any livestock species.

5. Conclusions

The steady increase in G6P and F6P concentrations, after 100 min postmortem, indicates that the postmortem decline in pH ultimately inactivates PFK, which halts glycogen depletion. When glycogen concentrations are above a certain threshold, we conclude that the rate of inactivation of PFK, not initial glycogen concentration, dictates ultimate pH in an economically important cut of meat.

CHAPTER V

SUMMARY

These data demonstrate that the inactivation of PFK mediated by the natural occurring decline in pH ultimately causes a buildup of G6P and F6P in postmortem bovine muscle. Additionally, the accumulation of G6P inhibits both glycogen phosphorylase and hexokinase, which limits further glycogen degradation. However, there were slight differences in the rate and extent of metabolism between the different muscles. The rate and extent of glycolysis appeared to occur more rapidly in the *M. longissimus* muscle, whereas the concentration of glycogen observed in the the *M. sternocephalicus* muscle at d 4 was achieved by 2 h postmortem in the *M. longissimus* muscle. Likewise, the concentrations of G6P and F6P measured at d 4 in the *M. sternocephalicus* muscle was seen by 6 h in the *M. longissimus* muscle when compared to the d 4 *M. sternocephalicus* muscle samples. These differences imply that while the regulation provided by PFK may be similar, there is metabolic variation between muscle types.

The application of glycolytic potential as a predictor of meat quality is based on the assumption that the concentration of glycogen is the primary determinant of ultimate pH. The apparent decline in glycolytic potential between the initial and final samples within both studies suggests that some oxidative metabolism of glucose carbon occurred in the muscle postmortem. This would contribute to the inaccuracy of glycolytic potential as a predictor of meat quality.

Reducing the pH to 7.0 severely depressed PFK activity, displaying readily measurable activity only at 1.0 mM F6P. Even at this substrate concentration, PFK activity was 50% lower than the activity observed at pH 7.8. The profound sensitivity of PFK to a small change in pH strongly indicates that it will be inactivated early postmortem. Any subsequent glycolysis proceeds at a lower rate, only by mass action of the elevated F6P.

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