The Increase of Short-term Heat Exposure Temperature at Extreme Range Decreased Phosphofructokinase-1 Activity and AMPK Phosphorylation in Postmortem Chicken Breast Muscle

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Abstract

Preslaughter exposure to high ambient temperature could accelerate postmortem glycolysis and impair chicken breast (*pectoralis major* muscle) quality. However, previous studies indicated that it might be different when the temperature of short-term heat exposure (SHE) raises to extreme range (above 38°C). Therefore, the objectives of this study were to evaluate the effect of SHE in the range of extreme temperatures (36°C, 38°C, and 40°C) on the activity of glycolytic enzymes and AMP-activated protein kinase (AMPK) in postmortem muscles. The activity of key glycolytic enzymes and phosphorylation of Threonine 172 in AMPK α subunit (p-AMPK-α[Thr172]) during early postmortem (0.1, 1, 2, and 4 h) and the muscle pH decline (0.1, 1, 2, 4, and 24 h) were evaluated. SHE did not affect ($P > 0.05$) glycogen phosphorylase a (GPa) and pyruvate kinase (PK) activity within 4 h postmortem. However, the phosphofructokinase-1 (PFK-1) activity and phosphorylation of AMPK-α[Thr172] decreased ($P < 0.05$) as antemortem SHE temperature increased from 36°C to 40°C, which could explain the faster pH decline in the 36°C group at 1 h and 2 h postmortem ($P < 0.05$), and it could be attributed to proteotoxic stress response resulting from extreme heat exposure. Hexokinase (HK) activity was also affected by the SHE temperature ($P < 0.05$) with the 36°C group having the highest ($P < 0.05$) activity within 4 h postmortem. The results of the current study, for the first time, indicated that the increase of antemortem SHE temperature at extreme range does not always accelerate the pH decline rate and increase postmortem glycolytic enzyme activity and AMP-activated protein kinase phosphorylation and that the postmortem metabolism is dependent on SHE temperature. Further research is necessary to explore the biochemical basis of this temperature-dependent pH decline, glycolytic enzyme activity, and AMPK phosphorylation.

Backgrounds

Pale, soft, and exudative (PSE) meat is a quality defect of chicken breast associated with abnormally light color, soft texture, and poor water holding capacity that has become one of the biggest challenges to the poultry industry worldwide [1–3]. The conversion of muscle to meat during the early postmortem period is a short but intensive period accompanied by severe changes in metabolism [4, 5]. Rapid glycolysis and pH decline during the early postmortem period is considered to be one of the major reasons for PSE meat [6].

AMP-activated protein kinase (AMPK) is an energy status sensor that maintains cellular energy homeostasis [7] and is a mediator of proteotoxic stress response [8]. The maximal AMPK activity is initiated by the phosphorylation of the amino acid Threonine 172 in the AMPK α subunit (p-AMPK-α[Thr172]) [9]. Previous studies have indicated that an increased expression of p-AMPK-α[Thr172] at early postmortem is associated with rapid glycolysis and pH decline at postmortem, which can lead to meat quality defects [10–15]. The activated AMPK can, in turn, accelerate postmortem phosphofructokinase-2 (PFK2) activity [16], an enzyme that catalyzes the production of fructose-2,6 bisphosphate (F-2, 6-BP) to further activate phosphofructokinase-1 (PFK1) [17–19].

The incidence of PSE could be increased by heat stress (exposure to high ambient temperature) in birds [20, 21], probably due to the altered metabolism, acid/base status, oxidative reactions, and changes in hormonal secretions [22]. Although several studies have examined the influence of acute heat stress ranging from 32 °C to 41 °C on chicken breast quality [21, 23–29], inconsistent results were observed when the antemortem acute heat stress ranged from 36 °C to 41 °C [21, 25, 28, 29]. Meat quality, such as cooking loss, color attributes, and water holding capacity in chicken breast were impaired by one-hour antemortem acute heat stress at 36 °C and 37 °C [21, 28] but not at 40 °C and 41 °C [25, 29].

Our recent study also indicated that poultry meat quality was affected by short-term heat exposure (SHE) of 36 °C, but exposure to temperatures above 38 °C did not always lead to more pale and lower WHC breast meat [30]. Moreover, the breast meat quality defects were reduced as heat exposure temperatures raised from 36 °C to 40 °C [30]. One possible explanation for this variation in meat quality with different exposure temperatures could be the change in of muscle metabolism postmortem [30]. However, the biochemical basis of this variation is not clearly understood. Therefore, the objectives of this study were to evaluate the changes in glycolytic enzyme activity and AMPK phosphorylation at early postmortem after antemortem short-term heat exposure (SHE) of 36 °C, 38 °C, or 40 °C.

Methods

Animal treatment and sampling

One hundred male broilers (from Arbor Acres) were raised under similar conditions (25 °C ± 1 °C, RH 58% ~ 62%) for the first six weeks. Twenty-four broilers with similar weight (2.32 ± 0.02 kg) were selected from those and eight broilers each (n = 8) were randomly assigned to either (1) 36 °C heat exposure, (2) 38 °C heat exposure, or (3) 40 °C heat exposure for 90 minutes. All 24 broilers were moved to a preparation room (25 °C) and caged individually for 24 hours before the SHE for adaption. After the adaptation period, the broilers were moved into a heat exposure room for applying the treatments for 90 min. All the cage positions in the heat exposure room were the same as in the preparation room, and water was offered throughout the treatment.

After 90 minutes of exposure, each broiler was moved out and then slaughtered and bled within five minutes. After exsanguination, boneless *pectoralis major* (PM) muscle from both sides of the bird was removed manually by knife cutting immediately, without scalding or defeathering, and stored at 4 °C for aging and analysis. Two grams of sample was collected from PM muscle from each side at 0.1 h, 1 h, 2 h, and 4 h postmortem for enzyme analysis or pH measurement. An additional sample from right breast muscle was collected at 24 h postmortem for the ultimate pH measurement. The collected samples were placed into 5 mL antifreeze tubes, frozen in liquid nitrogen, and stored at -80 °C until analysis.

Muscle pH value

The pH values of the right breast muscles were measured, according to Wang et al. [31]. One-gram muscle sample was homogenized in 9 mL of 5 mM iodoacetate solution for 60 s by a blender (IKA, T18,

Staufen, Germany) at medium speed. The pH was measured by a pH meter (SevenGo, Mettler Toledo, Zurich, Switzerland). The pH value was calculated from the average of the three repetitions.

Enzyme activity measurement

The activity of enzyme glycogen phosphorylase a (GPa) was measured because GPa is typically more active than glycogen phosphorylase b, and the latter form can be converted to GPa by phosphorylation [32] or calcium activation [33]. Glycogen phosphorylase a (GPa) activity was estimated spectrophotometrically using a glycogen phosphorylase assay kit (GPA-2-Y, Suzhou Comin Biotechnology Co. Ltd, Suzhou, China). Approximately 100 mg sample was homogenized in 1 mL extraction solution. After centrifugation (8,000 × g at 4 °C, 10 min), 10 μL of the supernatant was mixed with 10 μL distilled water and 180 μL reaction mixture in a microvolume quartz cuvette and held at 37 °C. The absorbance values at 340 nm were recorded after 5 min (A1) and 10 min (A2). Protein concentration (Cpr) was determined using a BCA protein assay kit (BCAP-2-W, Suzhou Comin Biotechnology Co. Ltd, Suzhou, China). The activity of GPa was calculated as (643 × A)/Cpr where A = A2 – A1.

Hexokinase (HK) activity was determined spectrophotometrically with a hexokinase assay kit (HK-2-Y, Suzhou Comin Biotechnology Co. Ltd, Suzhou, China). Approximately 100 mg of sample was homogenized in 1 mL extraction solution. After centrifugation (8,000 × g at 4 °C, 10 min), 30 μL of the supernatant was mixed with 1008 μL reaction mixture in a quartz cuvette and held at 37 °C. The absorbance values at 340 nm were recorded after 20 s (A1) and 5 min 20 s (A2). Protein concentration (Cpr) was determined using a BCA protein assay kit (BCAP-2-W, Suzhou Comin Biotechnology Co. Ltd, Suzhou, China). The activity of HK was calculated as (1113 × A)/Cpr, where A = A1 – A2.

Phosphofructokinase-1 (PFK1) activity was determined spectrophotometrically with a phosphofructokinase-1 assay kit (PFK-2-Y, Suzhou Comin Biotechnology Co. Ltd, Suzhou, China). Approximately 100 mg of sample was homogenized in 1 mL extraction solution. After centrifugation (8,000 × g at 4 °C, 10 min), 30 μL of the supernatant was mixed with 810 μL reaction mixture in a quartz cuvette and held at 37 °C. The absorbance values at 340 nm were recorded after 20 s (A1) and 10 min 20 s (A2). Protein concentration (Cpr) was determined using a BCA protein assay kit (BCAP-2-W, Suzhou Comin Biotechnology Co. Ltd, Suzhou, China). The activity of PFK1 was calculated as (450 × A)/Cpr, where A = A1 – A2.

Pyruvate kinase (PK) activity was determined spectrophotometrically with a pyruvate kinase kit (PK-2-Y, Suzhou Comin Biotechnology Co. Ltd, Suzhou, China). Approximately 100 mg of sample was homogenized in 1 mL extraction solution. After centrifugation (8,000 × g at 4 °C, 10 min), 50 μL of the supernatant was mixed with 950 μL reaction mixture in a quartz cuvette and held at 37 °C. The absorbance values at 340 nm were recorded after 20 s (A1) and 2 min 20 s (A2), respectively. Protein concentration (Cpr) was determined using a BCA protein assay kit (BCAP-2-W, Suzhou Comin Biotechnology Co. Ltd, Suzhou, China). The activity of PK was calculated as (2613 × A)/Cpr, where A = A1 – A2.

Immunoblot analysis for phosphorylation of AMPK

The determination of p-AMPK-α[Thr172] was modified based on previous methods [14, 34]. Frozen muscle samples (0.5 g) were homogenized (13,500 × g for 10 s) using a polytron homogenizer (IKA Works, Inc., Wilmington, NC, USA) in 5 mL pre-cooled buffer containing 20 mmol/L Tris-HCl (pH 7.4 at 4 °C), 2% sodium dodecyl sulfate (SDS), 5 mmol/L ethylenediamine tetraacetic acid (EDTA), 5 mmol/L ethyleneglycoltetracatic acid (EGTA), 1 mmol/L dithiothreitol (DTT), 100 mM sodium fluoride (NaF), 2 mmol/L sodium vanadate, 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL leupeptin and 10 μg/mL pepstatin. Muscle homogenates were then centrifuged at 17,500 × g at 4 °C for 20 min, and the supernatants were collected into fresh tubes. Protein concentration was determined by bicinchoninic acid (BCA) protein assay kit (Sigma, Dorset, UK).

For electrophoresis, each homogenate was mixed with an equal amount of 2 × standard sample loading buffer and heated for 3 min in a dry heater (100 °C). Equal amounts of total protein (45 μg) were resolved by electrophoresis (Bio-Rad, Richmond, CA, USA) on 10% SDS-PAGE (polyacrylamide gel electrophoresis) before being transferred electrophoretically to a polyvinylidenefluoride (PVDF) membrane. The transfer was done with a transfer buffer containing 25 mmol/L Tris, 192 mmol/L glycine, 15% (v/v) methanol, 0.01% (w/v) SDS using 90 V for 90 min at 4 °C. After transfer, the membranes were incubated in 5% non-fat dry milk in Tris-buffered saline with Tween-20 (TBST; 0.8% NaCl (wt/vol), 0.02% KCl (wt/vol), 0.24% Tris (wt/vol), 0.05% Tween-20 (vol/vol)) for 1 h. Primary antibody, phospho-AMPK-α[Thr172] antibody (Cell Signaling Technology, Beverly, MA, USA) or monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Bio-world, St. Louis Park, MO, USA) was diluted in TBST. The PVDF membranes were incubated overnight at 4 °C with the primary antibody. The blots were washed and incubated for 1.5 h with a secondary antibody (Jackson, West Grove, PA, USA). After 30 min washing, membranes were visualized using ECL Western blotting reagents (Amersham Bioscience, Piscataway, NJ, USA) and exposed to film (MR; Kodak, Rochester, NY, USA). The band densities were quantified by using Imager Scanner II and Image Quantity One software (GE, CT, USA). Band density among different blots was normalized according to the density of the reference band.

Statistical analysis

A split-plot design was used to evaluate the effects of antemortem SHE temperature and postmortem time on muscle pH, glycolytic enzyme activity, and AMPK phosphorylation. Samples were assigned to temperature x postmortem time combinations. Data analysis was performed by R (version 3.6.1) using the lme4 package as a mixed model, where temperature, postmortem time, and their interactions were fixed effects, and the random effect in the model was the individual bird. Differences between least-square means ($P < 0.05$) were determined by Tukey's multiple comparisons.

Results And Discussion

Muscle pH

The muscle pH during the early postmortem in response to SHE is presented in Fig. 1. There was an interaction between temperature and postmortem time ($P < 0.05$). All the three temperature groups had

same initial muscle pH at 0.1 h postmortem ($P > 0.05$), then the pH in 36 °C group rapidly dropped from 0.1 h to 1 h postmortem and had a lower pH than the 40 °C and 38 °C group at 1 h and 2 h postmortem ($P < 0.05$). However, no difference was observed in pH among different temperature treatments at 4 and 24 h postmortem. A similar pH decline and ultimate pH values after SHE have been reported in previous studies as well [29–31]. This indicated that the increase of SHE temperature from 36 °C to 40 °C caused a slower pH decline at early postmortem.

Glycolytic Enzyme Activity

The GPa activity during the early postmortem in response to SHE is presented in Fig. 2A. While there was no interaction between temperature and postmortem time ($P < 0.05$), a main effect of postmortem time ($P < 0.05$) was observed for the GPa activity. Overall, the GPa activity decreased ($P < 0.05$) from 0.1 h to 4 h. A similar trend of GPa activity in postmortem muscle has been reported by other researchers [6, 35], which could be attributed to the pH decline from 6.8, the optimum environment pH for GPa activity [36]. However, the GPa activity within 4 hours postmortem was not ($P > 0.05$) influenced by the SHE temperatures used in the current study. On the other hand, a main effect of temperature ($P < 0.05$) was observed on the HK activity (Fig. 2B). The HK activity at 36 °C was higher ($P < 0.05$) than the 38 °C group; however, there was no difference ($P > 0.05$) between HK activity of the 40 °C group and the 36 °C or 38 °C group. Moreover, the HK activity was influenced by the postmortem time ($P < 0.05$) with the activity increasing ($P < 0.05$) from 0.1 h to 2 h postmortem, followed by a decrease ($P < 0.05$) from 2 h to 4 h postmortem. A similar trend of HK activity in postmortem muscle has been reported by other researchers [35], which could be explained by the high residual activity of HK around pH 6.0 [37].

The PFK1 activity during early postmortem time in response to SHE is presented in Fig. 2C. Both temperature and postmortem time influenced ($P < 0.05$) the PFK1 activity, with the activity decreasing ($P < 0.05$) as time postmortem increased. The PFK1 activity in 36 °C group was higher ($P < 0.05$) than 40 °C group, but there was no difference between 38 °C group and 36 °C or 40 °C group. This result indicated that the heat stress temperature affected PFK1 activity at early postmortem, and PFK1 activity was reduced by the increase of antemortem SHE temperature from 36 °C to 40 °C. A similar temperature effect was also observed in a vitro system, which showed that PFK1 activity under 42 °C was lower than that under 33 °C [6]. Unlike PFK1, the PK activity at early postmortem was not ($P > 0.05$) influenced by the SHE (Fig. 2D). A main effect of postmortem time ($P < 0.05$) was observed with a decrease ($P < 0.05$) in PK activity from 0.1 and 1 h to 4 h postmortem, which could be attributed to the pH decline further from its optimum pH at mildly alkaline range [38, 39].

Phosphorylation of AMPK

The amount of p-AMPK-α[Thr172] during the early postmortem is shown in Fig. 3. Temperature influenced ($P < 0.05$) the amount of p-AMPK-α[Thr172] with the 36 °C group having greater ($P < 0.05$) phosphorylation than the 40 °C group, but there was no difference ($P > 0.05$) between 38 °C group and 36 °C or 40 °C group. This result indicated that the high temperature of antemortem SHE affected p-

AMPK-α[Thr172] at early postmortem, and the phosphorylation decreased with increase in antemortem SHE temperature.

In addition, the postmortem time also influenced ($P < 0.05$) the p-AMPK-α[Thr172]. While the phosphorylation increased ($P < 0.05$) from 0.1 h to 1 h postmortem, it decreased ($P < 0.05$) from 1 h to 2 h postmortem. In agreement, previous research also reported that p-AMPK-α[Thr172] in chicken PM muscle treated by antemortem transport stress increased from 0.5 h to 1 h, followed by a decrease to 4 h postmortem [11]. Moreover, similar trends in AMPK activity in postmortem muscle have been reported by other researchers [16, 40–44].

In the current study, changes in GPa, HK, and PK activity did not correspond with the changes in AMPK activation with different SHE. These results are in agreement with previous studies, which suggested that the AMPK activation cannot fully account for postmortem GPa [16, 35, 40, 43, 44] and PK activity [16, 40, 41]. However, our results demonstrated that the PFK1 activity corresponded with p-AMPK-α[Thr172] with both PFK1 activity and p-AMPK-α[Thr172] decreasing when temperature increased from 36 °C to 40 °C. These results are also in agreement with previous studies suggesting that AMPK regulates postmortem glycolysis through PFK1 [16]. However, a recent study indicated that PFK1 can be directly increased by AMP, and this activation is more feasible than the activation by the p-AMPK induced fructose-2,6 bisphosphate (F-2, 6-BP) production [45]. It is also possible that decreased p-AMPK-α and PFK1 activity in 40 °C as compared to 36 °C group resulted from the difference in AMP content, but this possibility cannot be confirmed in the current study and needs further investigation. In addition, recent research pointed that PFK1 activity can affect glycolysis and ultimate pH in postmortem chicken PM muscle [46]. There was no difference in ultimate pH among different temperature groups in the current study, but the 36 °C group had lower pH than other groups at 1 h and 2 h postmortem, which is possibly caused by increased PFK1 activity in the 36 °C group at early postmortem.

To the best of our knowledge, the current study is the first one to report a lower PFK1 activity and phosphorylation of AMPK-α[Thr172] in chicken PM muscle from birds exposed to SHE of 40 °C compared to 36 °C. This decreased p-AMPK-α[Thr172] and PFK1 activity in the 40 °C group could be attributed to proteotoxic stress response due to preslaughter extreme heat exposure, which can inhibit p-AMPK-α[Thr172] and activate heat shock factor 1 to increase the expression of heat shock proteins [8, 47], a set of proteins that can protect muscle cell from calcium overload, cell death signaling, and proteolysis and preserve meat quality [48]. In chicken PM muscle, heat shock protein 70 (Hsp70), a member of heat shock family, has been considered as a protective protein due to its higher abundance in normal meat than defective meat [49–51]. In support, previous research indicated that two-hour heat exposure under 40 °C could increase the expression of Hsp70 in chicken breast muscle to more than three times [52], which suggests that SHE of 40 °C might have increased the cellular defensive mechanism in chicken breast meat. Moreover, the chicken PM muscle after antemortem SHE of 40 °C exposure had better meat quality, less mitochondrial damage, greater protein solubility, and higher muscle fiber integrity as compared to SHE of 36 °C [30]. Some studies have indicated that Hsp70 can directly interact with PFK1 in chicken PM muscle during the early postmortem period [49] and have a negative correlation with p-AMPK-α[Thr172]

[13]. Hence, we believe that the increase of antemortem SHE temperature from 36 °C to 40 °C might have stimulated the proteotoxic stress response in chicken PM muscle during the early postmortem, which could cause the decreased PFK1 activity, lower p-AMPK-α[Thr172], and slower pH decline. However, further research is necessary to explore the mechanism behind the decreased postmortem p-AMPK-α[Thr172] and PFK1 activity due to antemortem extreme heat exposure.

Conclusions

The results from the current study demonstrated that antemortem SHE temperature could influence pH decline, AMPK activation, and glycolytic enzyme activity in chicken PM muscle at early postmortem. Specifically, the early postmortem PFK1 activity and p-AMPK-α[Thr172] decreased as antemortem SHE temperature increased from 36 °C to 40 °C, which could explain the faster pH decline in the 36 °C group at 1 h and 2 h postmortem, and it could be attributed to proteotoxic stress response resulting from extreme heat exposure. HK activity was also affected by the SHE temperature with the 36 °C group having the highest activity within 4 h postmortem. The SHE temperatures used in the current study did not influence the GPa and PK activity within 4 h postmortem. These results also for the first time indicated that the increase of antemortem SHE temperature does not always increase the pH decline rate, postmortem glycolytic enzyme activity, and AMP-activated protein kinase phosphorylation and that the postmortem metabolism is dependent on SHE temperature. The mechanisms behind this temperature-dependent pH decline, glycolytic enzyme activity, and AMPK activation need further investigation.

Declarations

Ethical approval and consent to participate

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Shandong Agricultural University (No. 2001002) and performed in accordance with the “Guidelines for Experimental Animals” of the Ministry of Science and Technology (Beijing, China). All efforts were made to minimize the suffering of the animals.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

Conceptualization, Rongrong Liang; Methodology, Mingyue Zhang and Minghao Zhang; Formal Analysis, Mingyue Zhang; Investigation, Mingyue Zhang and Chaoyu Zhai; Resources, Hai Lin; Data Curation, Mingyue Zhang; Writing – Original Draft Preparation, Mingyue Zhang and Chaoyu Zhai; Writing – Review & Editing, Xin Luo, Minghao Zhang, Lixian Zhu, Mahesh N. Nair, and Rongrong Liang; Visualization, Chaoyu Zhai; Supervision, Xin Luo; Project Administration, Rongrong Liang; Funding Acquisition, Rongrong Liang.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

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Figures

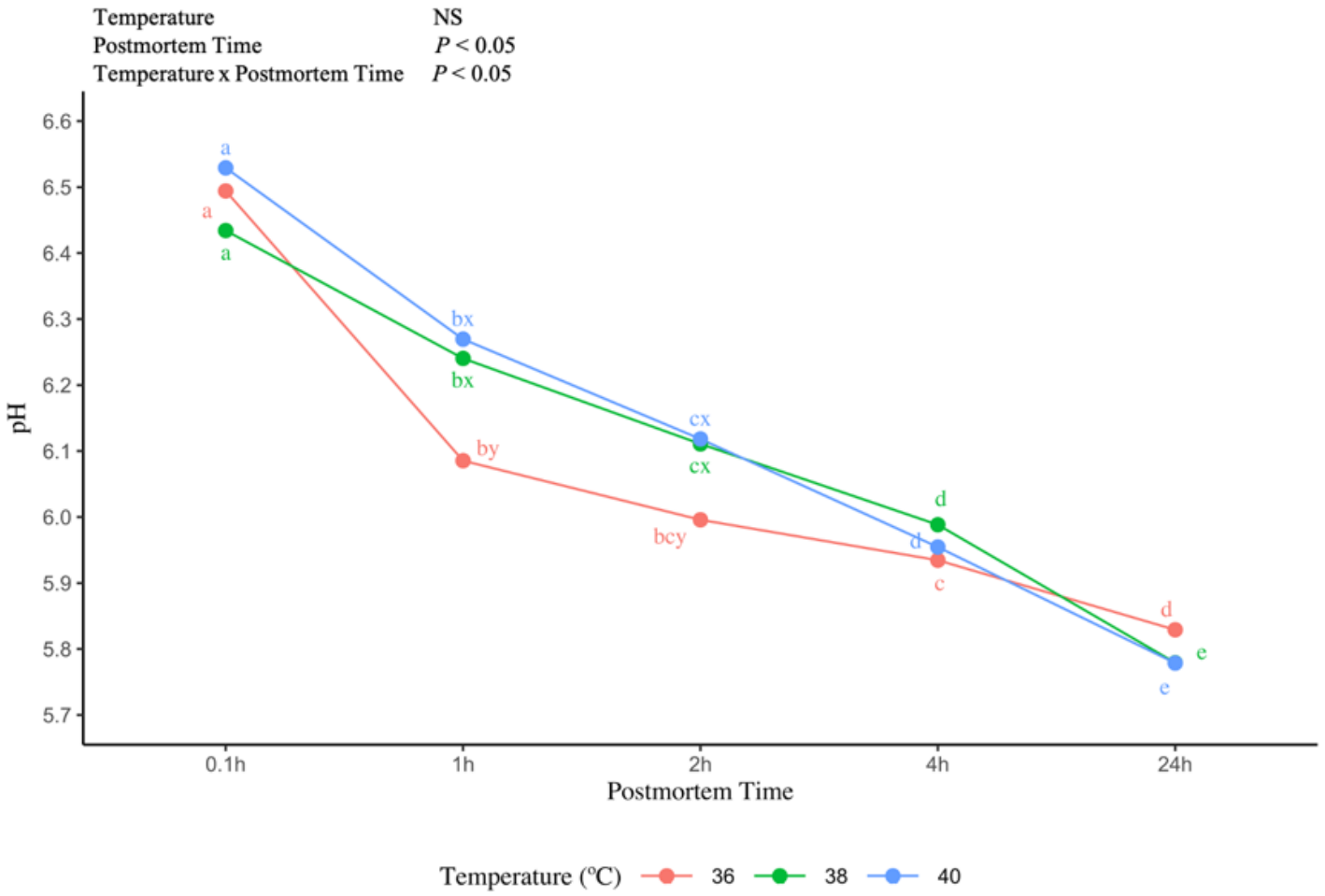

Figure 1

Effect of antemortem short-term heat exposure (SHE) and postmortem time on pH decline (n=8) in chicken pectoralis major muscle. Least square means for the pH of different temperature groups at same postmortem time without a common letter (x-y) differ (P < 0.05). Least square means for the pH of same temperature group at different postmortem time without a common letter (a-c) differ (P < 0.05). Standard error for comparison = 0.0315. Abbreviations: NS = no significance.

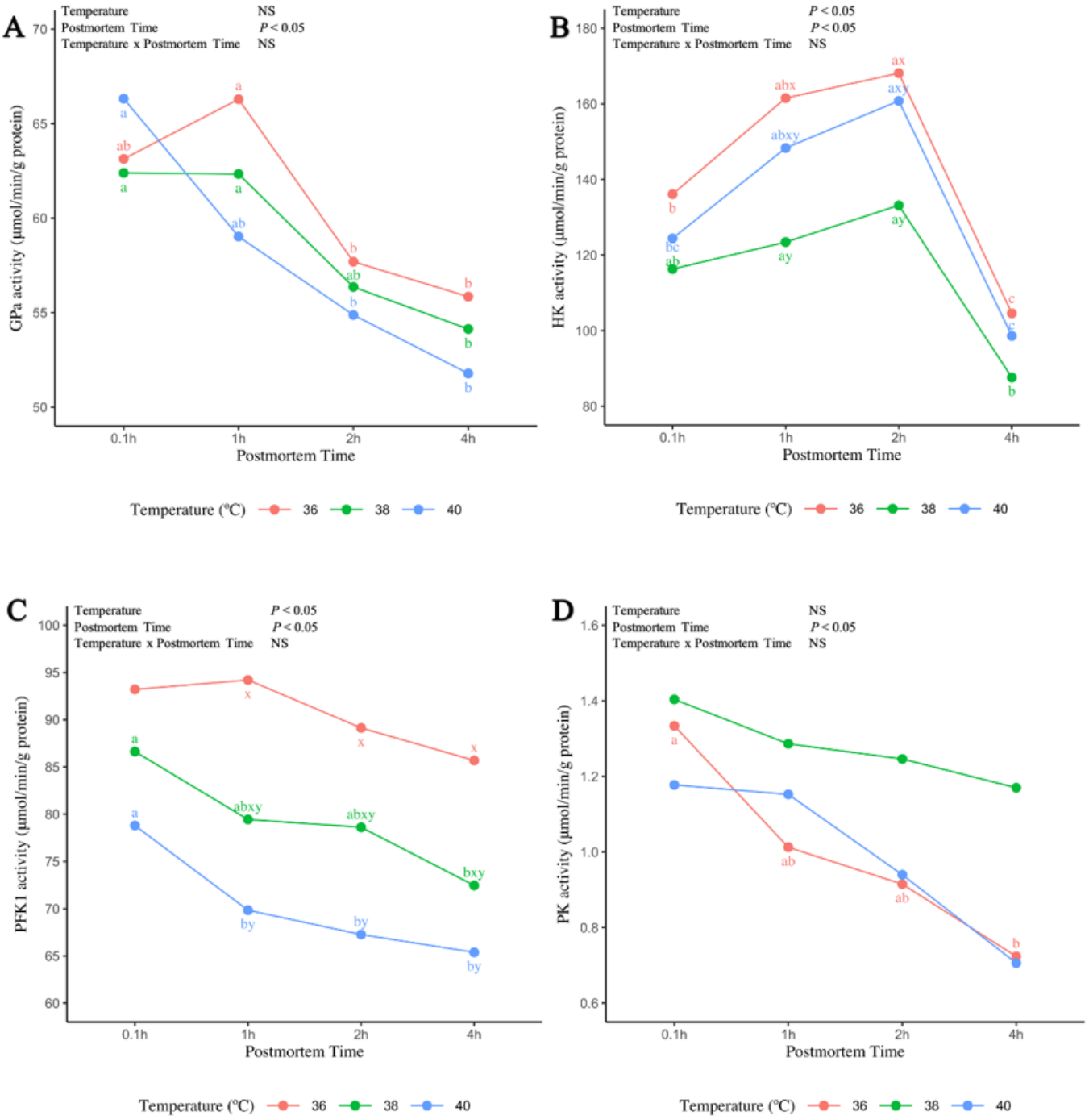

Figure 2

Effect of antemortem short-term heat exposure (SHE) and postmortem time on glycolytic enzyme activity (n=8) in chicken pectoralis major muscle. For each enzyme, least square means for the enzyme activity of different temperature groups at same postmortem time without a common letter (x-y) differ (P < 0.05). Least square means for the enzyme activity of same temperature group at different postmortem time without a common letter (a-c) differ (P < 0.05). Standard error for the activity of each enzyme: GPa=5.3,

HK=9.51, PFK1=5.09, and PK=0.171. Abbreviations: GPa = glycogen phosphorylase a; HK = hexokinase; PFK1 = phosphofructokinase-1; PK = pyruvate kinase; NS = no significance.

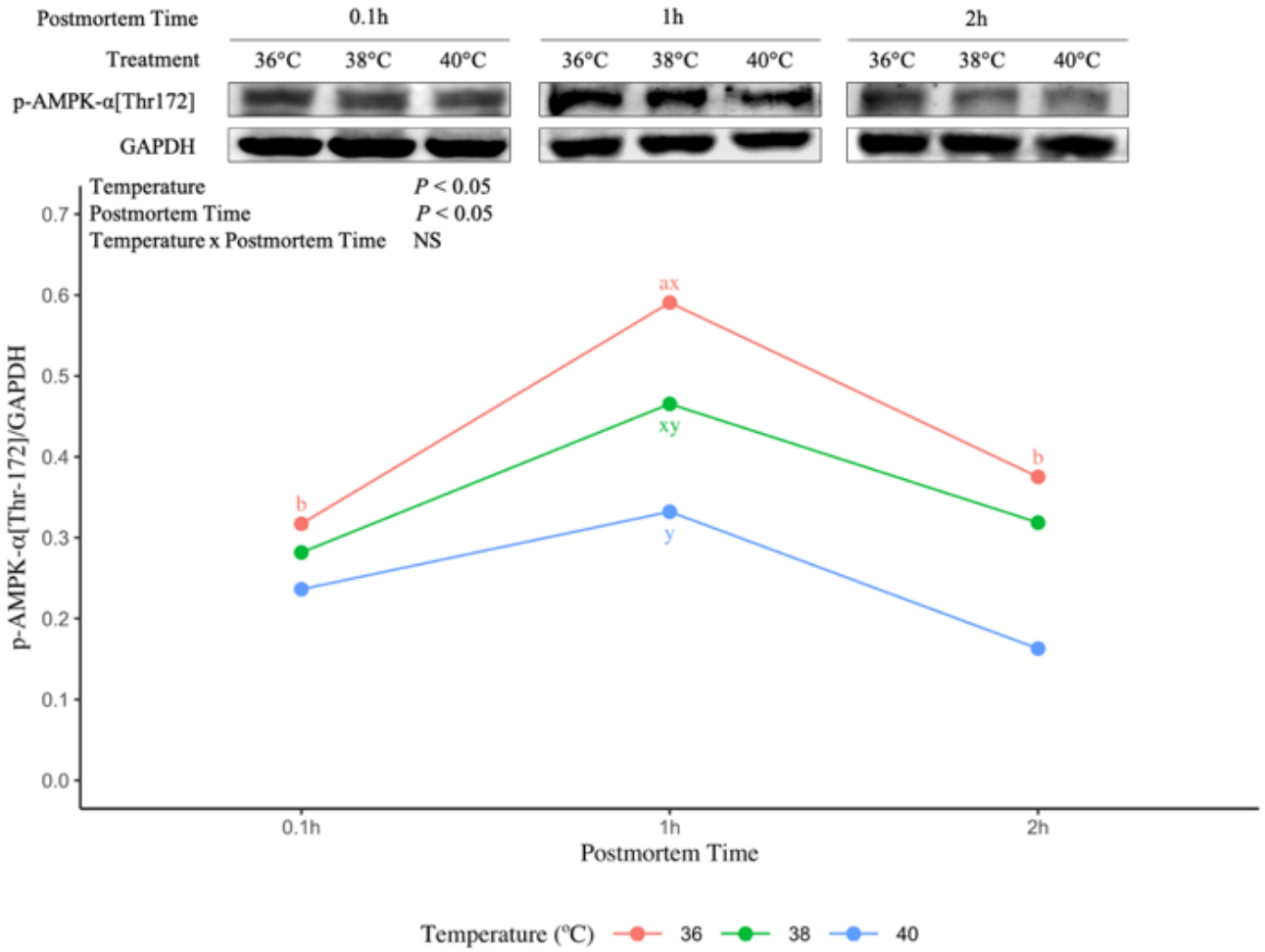

Figure 3

Effect of antemortem short-term heat exposure (SHE) and postmortem time on phosphorylation of Thr172 in AMPK α subunit (p-AMPK-α[Thr172]; n=8). Least square means for the p-AMPK-α[Thr172] of different temperature groups at same postmortem time without a common letter (x-y) differ (P < 0.05). Least square means for the p-AMPK-α[Thr172] of same temperature group at different postmortem time without a common letter (a-b) differ (P < 0.05). Standard error for comparison = 0.0648. Abbreviations: GAPDH = glyceraldehyde-3-phosphate dehydrogenase; NS = no significance.