Effects of Heat Stress on Porcine Skeletal Muscle Metabolism

By,

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STATEMENT BY AUTHOR

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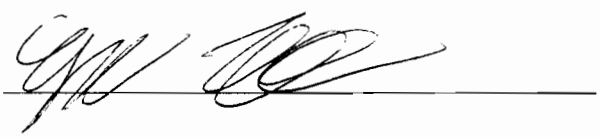
Signed: 

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Statement of Purpose:

Evidence indicates that mammals, particularly agricultural species such as swine, experiencing heat stress undergo dramatic changes in energy metabolism that may affect their heat tolerance, health and production parameters, such as lean tissue accretion. The purpose of this study was to determine the effects of heat stress on the skeletal muscle metabolism of growing pigs, specifically with respect to carbohydrate metabolism. Investigation into the interactions between heat stress, skeletal muscle metabolism and fuel selection will not only yield a clearer understanding of the underlying biology but may lead to useful applications within the agricultural industry as well as the human biomedical sector.

Statement of Relevance:

It is estimated that the losses due to heat stress cost the U.S. agricultural industry close to one billion dollars annually (St-Pierre et al., 2003). Considering that heat stress is a global problem, the cost to the worldwide agriculture reaches staggering proportions. Therefore, studies aimed at understanding the effects of heat stress on animal health, metabolism and production are timely. This is especially true if they result in interventions that positively affect animal health and production and limit costs incurred with heat stress abatement.

Methodology

Animals and Design

This particular study is associated with a funded project between Iowa State University and University of Arizona. It is part of a M.S. thesis where complete experimental description can be found (Pearce, M.S. Thesis, Iowa State University, 2011). Iowa State University Institutional Animal Care and Use Committee approved all procedures involving animals. Female crossbred gilts (n = 48, 35 ± 4 kg BW) were selected by body weight and housed in individual pens (with individual feeders and waters) in one of two rooms (24 pens/room) at thermal-neutral conditions. Animals were allowed to acclimate to their environment for 5 d at the Iowa State University Swine Nutrition farm prior to start of the experiment. Pigs were assigned to one of three environmental treatments: 1) thermal-neutral (TN) conditions (20°C; 35-50% relative humidity) with ad libitum feed intake, 2) heat stress (HS) conditions (35°C; 20-35% relative humidity) with ad libitum intake or 3) TN conditions but pair-fed (PFTN) to mirror the nutrient intake of the HS pigs. To evaluate the temporal response to environment, pigs in the TN (n=18) and HS (n=24) conditions were sacrificed at 1, 3 and 7 d post initiation of environment treatment. The PFTN pigs (n=6) were only sacrificed at 7 d post initiation of nutrient restriction.

After environmental initiation, reduced feed intake in the HS pigs was calculated daily based on the percentage decrease from each animal's average feed intake prior to HS; the amount offered to PFTN pigs was also reduced by that amount. The PFTN group lagged one day behind the 7 d heat stress pigs in order to

calculate and implement feed intake reductions. Pair-feeding was used to eliminate confounding effects of dissimilar feed and nutrient intake. Individual animal feed intake was determined daily at 0800 h. Pair-fed pigs were fed calculated amounts thrice daily at (0700, 1200, and 2000 h) in an attempt to reduce large post-prandial shifts in carbohydrate and lipid metabolism.

A data recorder (Lascar® model EL-USB-2-LCD, Erie, PA) monitored room temperature and humidity with measurements occurring every 30 min. Ambient temperature in each room was controlled but humidity was not governed. All pigs were monitored continuously for signs of distress. Body temperature parameters were obtained four times daily (0800, 1200, 1600, and 2000 h). Rectal temperatures were recorded with a digital thermometer (Top care®, Waukegan, IL), skin temperatures at the shoulder and ham were recorded with an infrared temperature gun (Extech® instruments Model 42505, Waltham, MA) and respiration rates (breaths/min) calculated with a stopwatch.

Animals were sacrificed using the captive bolt technique followed by exsanguination. Skeletal muscle (psoas major) was harvested within 12 min of death. Tissue samples were snap-frozen in liquid nitrogen and stored at -80°C until further analyses.

Analysis of Gene Expression

Analysis of gene expression was performed as described previously (Rhoads et al., 2011). Briefly, total RNA was extracted using the Trizol reagent according to the manufacturer's protocol (Invitrogen; Carlsbad, CA) and was DNase treated (RNase-Free DNase set, Qiagen Inc., Valencia, CA) and purified by affinity chromatography (RNeasy®, Qiagen Inc.) to remove potential genomic DNA contamination. The total RNA concentration was determined by absorbance at 260 nm, and quality was verified the Experion System (Bio-Rad Laboratories, Inc.). Total RNA was stored at -80°C until gene expression analysis.

Total RNA (2 µg) was reverse transcribed using the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen). Primer sets for porcine genes were designed using Primer Express Software (Applied Biosystems) based on published porcine nucleotide sequences and are presented in Table 1. Reactions (25 µL) were prepared according to the manufacturer's instructions using the iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA), 100 pM of each forward and reverse primer, and 10 ng of cDNA. PCR quantification of each sample was performed in triplicate and SYBER Green fluorescence was quantified with the iQ5 Real Time PCR Detection System (Bio-Rad Laboratories, Inc.). Each assay plate contained negative controls and a standard curve (five serial dilutions of a pool cDNA sample) to determine amplification efficiency of the respective primer pair. For each assay 40 PCR cycles were run, with each cycle consisting of three stages (50°C for 2 min, 95°C for 10 min and 60°C for 1 min) and a dissociation curve was

included to verify the amplification of a single PCR product. Analyses of amplification plots were performed with the iQ5 Optical System Software version 2.0 (Bio-Rad Laboratories, Inc.).

Statistics

All data were statistically analyzed using the PROC MIXED procedure of SAS version 9.1 (SAS Inst. Inc. Cary NC). Data are reported as LSmeans and considered significant if $P < 0.05$. Data were evaluated using two distinct models. For daily measurements (body temperatures, respiration rates and feed intake) each animal's respective parameter was analyzed using repeated measures with an auto-regressive covariance structure and day as the repeated effect. The model included environment, day, and the interaction. Analysis also tested differences between all three environments on (TN, PFTN, HS) d 7 variables (obtained at sacrifice).

Literature Review:

Human relevance

Heat related illness represents a significant portion of sickness and death for humans. The Arizona Department of Health Services reported nearly 800 admitted cases in Arizona hospitals due to heat-inflicted illness each year. Heat causes a variety of illness in humans depending on the severity and length of exposure. The symptoms range from headache and thirst to renal failure and death. The human body has a narrow range of acceptance for inner heat variability, due to the specific

conditions of the cell that are necessary for optimal enzymatic reactions (Sanders 25).

The human body has adapted to efficiently and effectively regulate normal and regular environmental heat stress. The body continually rids itself of the heat waste produced due to muscle contractions, normal cellular processes, and homeostasis regulation. At environmental temperatures below the normal body core temperature (37ºC), the heat is dissipated into the atmosphere through radiation, convection and conduction of heat into the environment. Perspiration aids in the heat loss process within the body's thermal-neutral temperature range of 28-32ºC, depending on the humidity (Hajat, O'Connor, and Kosatsky, 2010). Once the environment temperature reaches or exceeds 40ºC, the body cannot cope with the stress using normal processes and may begin to undergo heatstroke. If the body is not cooled down sufficiently through cold fluid ingestion or relocated to a cooler environment in enough time, severe organ damage or complete organ failure can occur (Mayo Foundation for Medical Education and Research 2009).

Heat related illness develops in stages that increase in severity based on the length of heat exposure, environmental heat temperature intensity and stage of dehydration. The Office of Environmental Health at the Arizona Department of Health Services classifies heat stress into four major symptoms and illnesses. The first stage of heat illness is mild dehydration, indicated by thirst. If the victim is not administered water or removed from the heat, the heat stress escalates and heat cramps may occur in the form of muscle pain and spasms in the abdominal area or

legs. Heat cramps are the result of water and salt loss from heavy sweating. If the body fails to cool down further, the victim progresses to the next stage, called heat exhaustion. This is an indication that perspiration has failed to cool down the body to normal temperatures. Often, high environmental humidity or layers of clothing trap perspiration close to the body so it cannot evaporate and cool the skin. At this point, the body conserves its remaining fluids by sending them to the vital organs. The victim experiences heat exhaustion in the form of shock with the following symptoms: moist, pale skin, headache, nausea, and dizziness/weakness. The final stage of heat illness is heat stroke. This means the body's cooling system has shut down and the core body temperature may rise to dangerously high temperatures (>105ºF) that eventually cause brain damage or death. The victim becomes disoriented or confused and may experience hallucinations, especially of water. The body will conserve its remaining fluids for the vital organs. The following additional visible symptoms may be observed: vomiting, rapid pulse, shallow, rapid breathing, seizures, and loss of consciousness.

Treatment of heat related illness is simple at the earlier stages, but requires medical attention when the illness reaches the point of heat stroke. The Arizona Department of Health Services recommends relocating the victim to a cool place where they may comfortably rest and drink water in half glass doses every fifteen minutes. If the victim is unable to drink water or displays more symptoms of heat stroke, professional medical attention is needed as quickly as possible. At any stage of heat related illness, the body may be cooled topically by applying cool, wet fabrics

to the skin or placing ice packs on areas with large blood flow. A location in air conditioning or in front of a fan is best.

Luckily, heat related illness is quite preventable. The principle consequences from heat stress stem from fluid loss and core body temperature elevation. Additional fluid intake is necessary if spending time outdoors. The Arizona Department of Health Services recommends 1-2 liters of fluid intake for adults per hour outside. Sports drinks can replace salts lost through perspiration. Environmental heat stress may be avoided by wearing lightweight clothing to protect from the sun and by going outside during the coolest hours of the day, normally early morning before 7am. The Arizona Department of Health Services recommends avoiding protein heavy meals because digestion creates additional metabolic heat.

The principle responses to heat stress in humans can be classified into physiological and cellular. The most visible symptoms of heat stress are body fluid loss, or sweating. Other less visible processes are redistribution of blood away from the core of the body, increased heart rate and increased blood temperature (Sanders 26). The body continues these three major techniques to lower core body temperature until the core temperature reaches 40ºC. On the cellular level, heat alters the specific microenvironment within the cell such as pH and temperature. Those changes disrupt the weak interactions required reactions for normal function, such as protein folding, translation, and protein synthesis (Streffer, 1988). Other molecular effects of heat stress are change of lipid composition and change of lipid

fluidity (Bowler et al., 1973; Yatvin et al., 1982). . This study focuses on the cellular level response, particularly the gene expression.

The topic of heat stress is particularly relevant to Arizona because of the extreme desert climate. Temperatures in the summer rise to dangerous levels that are difficult for humans to tolerate. Such conditions in the summer pose life-threatening challenges to human migrants crossing the southern Arizona desert into the United States seeking economic opportunity. The journey is particularly dangerous for these groups because often there is no opportunity to treat the more minor heat problems like heat cramps and heat exhaustion before they escalate into full heat exhaustion. Before the onset of heatstroke, simple cooling techniques like seeking shade, drinking cool liquids, and rest can relieve heat related illness (Mayo Foundation for Medical Education and Research 2009). However, fear of the *migra* or border patrol and *coyote* pressures to keep up the pace of the group leave little opportunity for even brief pauses. The results of this study will further explain the experiences of those crossing the treacherous desert by working to explain the biological response to the environmental conditions inflicted on their bodies. Additionally, the results of this study may provide a clearer explanation of all mammalian response to heat stress and improve treatment of human patients and animals seeking treatment.

Animal modeling and relevance:

The pig model was particularly selected because of the traceable pig response to heat. Pigs are easier to study because their bodies heat in ways which are measurable, and retain heat due to less efficient cooling systems. Other animals, including humans, sweat in order to reduce their body temperature, but pigs lack functional sweat glands (Curtis, 1983) and instead use thermal panting to reduce their core body temperature (Patience et al, 2005). Pigs have relatively small mouths, which makes their panting ineffective. They cannot tolerate temperatures above 95ºF with 65% humidity (Reece, 2009). Additionally, pigs have a significant layer of subcutaneous fat, which also retains heat (Mount et al., 1979). The accumulation of these factors reduces their ability to efficiently cool down, and therefore the core temperature is more reflective of the outside environment. In the wild, pigs adjust for all of these factors by changing their own environment, like finding shade or pools of water. However, domesticated pigs do no have this option and cannot compensate for their unaccommodating physiology (Huynh, 2005). These combined factors make pigs an attractive model for heat stress studies.

The pig physiology that make the heat stress changes easy to track, also pose problems in translating the findings back to applicable suggestions for heat stress treatment in humans. However, the gene expression changes and metabolic effects of heat stress may be more similar to humans, and applicable to study populations under chronic heat conditions.

An important factor in the study is the control of feed intake. Previous studies have indicated that heat stress causes a decrease in feed, up to 50%, which poses an additional variable in the metabolic puzzle (DeShazer et al., 2009). However, previously the role of the decreased feed factor was unclear, compared to other variables. In this study, a separate category of pigs that have been pair-fed under thermo neutral conditions will be included to differentiate between the effects of heat stress and the effects of decreased feed intake.

The results of this study may have direct applications to industry practices and agricultural markets. For example, transportation of pigs is limited until night and pigs are often “hosed with water” due to their intolerance for heat (Reece, 2009). Further research on the direct effects of heat stress on pigs may create breakthroughs to both more efficiently and humanely care for pigs especially during the summer months.

Additionally, heat stress has been demonstrated to have a significant effect on the growth of pigs and their body composition, which alters their market value. Heat stress is even cited to be “one of the costliest issues in the US pork industry” at a rate of $300 million dollars annually (St. Pierre et al., 2003). It has been clearly proven that the body composition of pigs in heat stress conditions has more adipose tissue and less muscle mass, which decreases the market value (Collin et al., 2001). The applications of this heat stress hold the potential to alter practices in the swine industry and increase profits.

Energy requirements of animals support numerous basic processes including the mechanical work for muscle contraction and cell movement, active transport of molecules and ions across cell membranes and the synthesis of macromolecules and other biomolecules required by the cell (Himms-Hagen, 1976). Cellular energy requirements are met by the metabolism of fuel substrates including glucose, lactate, fatty acids and amino acids, through a series of highly integrated chemical reactions. Depending on cellular need, energy substrates can be converted into a number of metabolic intermediates and used in synthesis reactions, or utilized for the production of adenosine triphosphate (ATP). Generating ATP occurs in three stages including glycolysis, the tricarboxylic acid cycle (TCA) and oxidative phosphorylation, a process of the electron transport chain. Regulation of energy metabolism is complex involving several mechanisms including the regulation of synthesis and degradation rates of key enzymes, allosteric interactions and covalent modification of those enzymes, and the regulation of substrate into cells and intracellular compartments (Campbell, 1999; Berg et al., 2007). The energy charge of the cell (i.e. the relative amounts of ATP, ADP and AMP) plays a critical role in these regulatory processes determining the relative balance of anabolic and catabolic reactions by high and low net energy charges, respectively.

The regulation of glucose metabolism occurs during uptake into the cell, anaerobic glycolysis and pyruvate entry into the TCA cycle for oxidative metabolism (Sugden and Holness, 2006). The glycolytic pathway yields a comparatively small amount of ATP while continued oxidation of pyruvate by the TCA cycle produces the

greatest amount of ATP from remaining potential energy. Decarboxylation and association with coenzyme A to form acetyl-CoA is a prerequisite for entry of pyruvate into the TCA cycle. This reaction (conversion of pyruvate to acetyl-CoA), catalyzed by the pyruvate dehydrogenase (PDH) complex, is irreversible and therefore represents a critical site of glucose homeostasis (Randle, 1986; Harris et al., 2002). The PDH complex is subject to covalent modification by several isoforms of kinases and phosphatases, whose activities are affected by the intracellular energy status and transcriptional regulation. Phosphorylation of PDH (E1α) by various pyruvate dehydrogenase kinases (PDKs) inactivates the complex, while dephosphorylation by pyruvate dehydrogenase phosphatases (PDPs) activates the complex (Harris et al., 2002; Huang et al., 2002; Schummer et al., 2008). Four PDK isozymes and 2 phosphatase isozymes are expressed in varying amounts in mammalian tissues (Bowker-Kinley et al., 1998; Huang et al., 1998). PDK4 (the main isoform responsible for inactivating PDH) is predominately regulated at the transcriptional level in a stimulatory manner by glucocorticoids (Huang et al., 2002), free fatty acids (Huang et al., 2002) and peroxisome proferator-activated receptor (PPAR)-γ-coactivator-1α (PGC-1α), and inhibited by insulin (Huang et al., 2002).

Pyruvate dehydrogenase complex inactivation by elevated PDK4 protein (Wu et al., 1998; Wu et al., 1999; Sugden et al., 2000) occurs in many tissues (i.e. muscle, liver) in catabolic states, such as starvation, as a means of conserving 3 carbon molecules (pyruvate and lactate) for gluconeogenesis (Hutson and Randle, 1978; Fuller and Randle, 1984; Sugden et al., 1999). The products of the PDH

reaction, acetyl CoA and NADH, directly inhibit PDH activity and also indirectly inhibit PDH by increasing the activity of PDKs, while the substrates of PDH, pyruvate and ADP, increase PDH activity and inhibit PDK activity (Roche et al., 2001). The PDH complex is a target of substrate competition between glucose and fatty acids since their metabolism produces common intermediates (acetyl-CoA and NADH) that stimulate PDK activity and inhibit PDH activity (Sugden et al., 2001). In skeletal muscle, several days of a high fat diet increases PDK4 transcript levels (Chokkalingam et al., 2006) since β-oxidation of fatty acids produces high quantities of acetyl-CoA and NADH, therefore preventing glucose oxidation. Inactivation of the PDH complex prevents the conversion of pyruvate to acetyl-CoA, allowing pyruvate to instead be used for other purposes (i.e. conversion to lactate or alanine for utilization in gluconeogenesis). On the other hand, when glucose supply is high, cirtrate accumulates, which serves as a precursor for malonyl-CoA formation and inhibits carnitine palmitoyltransferase 1 (Holness and Sugden, 2003).

Concomitant with the onset of cellular stress is the altered expression of a protein family known as the heat shock proteins (HSPs). Members of this protein family are ubiquitously expressed across species and present at low levels in cells under normal conditions, but their levels increase greatly but transiently upon cellular insult (Li et al., 1983; Moseley, 1997). Heat shock proteins are classified based on their molecular weight (Feige and Polla, 1994; Westman and Sharma, 1998) as well as by structure and function (Park et al., 2000). Major HSPs in mammalian cells include HSP 110, 90, 70, 60 and 27 (Arya et al., 2007), each having separate

functions and cellular locations (Feige and Polla, 1994). Of these, HSP70 is known to play a critical role in cryoprotection and is frequently used as a biomarker of cellular stress (Arya et al., 2007). Expression levels of HSP70 are most closely correlated to the magnitude and duration of a thermal stress (Mizzen and Welch, 1988). Classically known as molecular chaperones, HSPs bind to unfolded or misfolded proteins and help restore their native conformation (Buchner, 1996; Bouchama and Knochel, 2002).

Results:

Irrespective of day or time of day, pigs exposed to HS had an increase (P<0.01) in rectal temperature (39.3 vs. 40.8°C) compared to TN pigs and this increase was maintained throughout the duration of the experiment. Rectal temperatures in PFTN pigs were decreased (P<0.05) compared to TN pigs by d 1 and this difference in body temperature was maintained throughout all 7 d (Figure 1). Independent of time of day, compared to TN and PFTN pigs, HS animals had an immediate increase (P<0.05) in respiration rate on d 1 (49 vs. 136 bpm). Respiration rates decreased (P<0.05) in HS pigs on d 2 and 3, but remained elevated (P<0.05) compared to TN and PFTN pigs (55 vs. 115 bpm) throughout d 7 (Figure 1).

TN pigs consumed 1.96 kg/d of feed and this did not differ (P>0.1) over time (Figure 2). Compared to TN pigs, HS pigs had an immediate decrease (47%;

P<0.05) in FI by d 1 and this extent of decrease continued through d 7. By design, PFTN nutrient intake pattern mirrored intake of the HS group (Figure 2).

PDH is an enzyme complex located in mitochondria necessary for glucose entry into the TCA cycle, and PDK4 is a kinase responsible for the inactivation of this complex. Within psoas skeletal muscle and compared to TN control, levels of PDK4 mRNA were increased ($P < 0.05$) in pigs exposed to heat at 24 hours although this effect was lost at subsequent 3 and 7 day timepoints (Figure 3). In contrast, PFTN animals exhibited a dramatic increase over both TN and HS animals at 7 days (Figure 3). As expected, pigs experiencing HS conditions displayed elevated HSP70 gene expression although this response tended to decline with advancing duration of HS exposure (Figure 4).

Analysis/Conclusion:

Cellular exposure to stressful situations, such as elevated ambient temperatures, causes an elevation in stress response proteins, called heat shock proteins (Currie and White, 1983; McArdle and Jackson, 1996; Liu et al., 2006). In this study, swine exposed to short duration heat load showed increased cytosolic HSP70 mRNA abundance in psoas skeletal muscle. However, this effect appeared to be lost as the duration of heat exposure lengthened. Several possibilities may help explain this unexpected finding. First, this may be a display of acclimation by the swine to the HS environment. Second, this may be likely due to animal-to-animal variation in heat response as SEMs (even among control animals) for mRNA

abundance was high. Previous studies indicate variation in thermal tolerance may be significant among individuals (Furuyama, 1982; Leon et al., 2005). A third possibility may relate to a combination of the magnitude and duration of the heat load. For example, many heat stress studies utilizing rodent models subject the animals to a much greater heat load (41 or 42°C) but for shorter duration (Hall et al., 2000; Ruell et al. 2004). Finally, reasons for the apparent lost expression upon heat exposure in the current study are unclear, but may suggest our experimental design may have increased stress levels (and therefore HSP abundance) in thermal neutral controls as well, either due to unknown factors such as transport stress, or placement in the environmental chamber without previous acclimation.

Several studies report that lactate efflux (presumably by skeletal muscle) as well as plasma lactate concentrations were increased during exercise in the heat (Fink et al., 1975; Angus et al., 2001), porcine malignant hyperthermia (Hall et al., 1983), and growing steers (O'Brien et al., 2010) indicating the possibility of an increase in anaerobic glycolysis. Since PDKs are responsible for the inactivation of the PDH complex and are regulated by intracellular energy status, metabolism intermediates (acetyl-CoA and NADH) as well as by stress hormones such as cortisol (Sugden et al., 2001; Sugden and Holness, 2006; Chokkalingam et al., 2006), it remains likely that altered cellular glucose metabolism is occurring during periods of HS. A similar stimulatory effect of HS on rodent skeletal muscle PDK4 gene expression was reported (Sanders, 2010, Ph.D. Dissertation, University of Arizona). It is possible that higher skeletal muscle PDK4 mRNA abundance in these

studies regardless of heat or nutrient stress may be due to glucocorticoid activation of PGC-1α/FOX01, a pathway leading to enhanced PDK4 transcription (Huang et al., 2002), since cortisol is increased during times of stress, presumably including that imposed by hyperthermia or nutrient restriction.

Exposure of growing swine to heat stress increased body temperatures and altered psoas skeletal muscle gene expression patterns relating to cellular energy use. In response to elevated temperatures, cellular defense increased the abundance of HSP70 presumably to protect the accumulation of damaged and mis-folded proteins. Moreover, data from the current experiment suggests that pigs exposed to a short duration heat load or a long duration nutrient restriction experience decreased capacity for complete glucose oxidation within skeletal muscle as evidenced by elevated PDK4 mRNA abundance. Future studies will need to determine rates of glucose oxidation as well as other fuel substrates within skeletal muscle during temperature and nutrient stresses.

Table 1. Oligonucleotide primers used for real-time PCR analysis (SYBR Green assay).

Transcript	Sequence[a]	Reference
HSP70	F: AGCACAAGAAGGACATCAGC	Kim et al., 2010
	R: GAAGTCGATGCCCTCGAACA	
PDK4	F: GCCTCAGTGGCATAAAAACC	Thaller et al., 2009
	R: CAGTGGTGGTAATACAAAGG	

[a]For each transcript, the sequence of the forward (F) and reverse ® primer is given in the 5′ to 3′ direction.

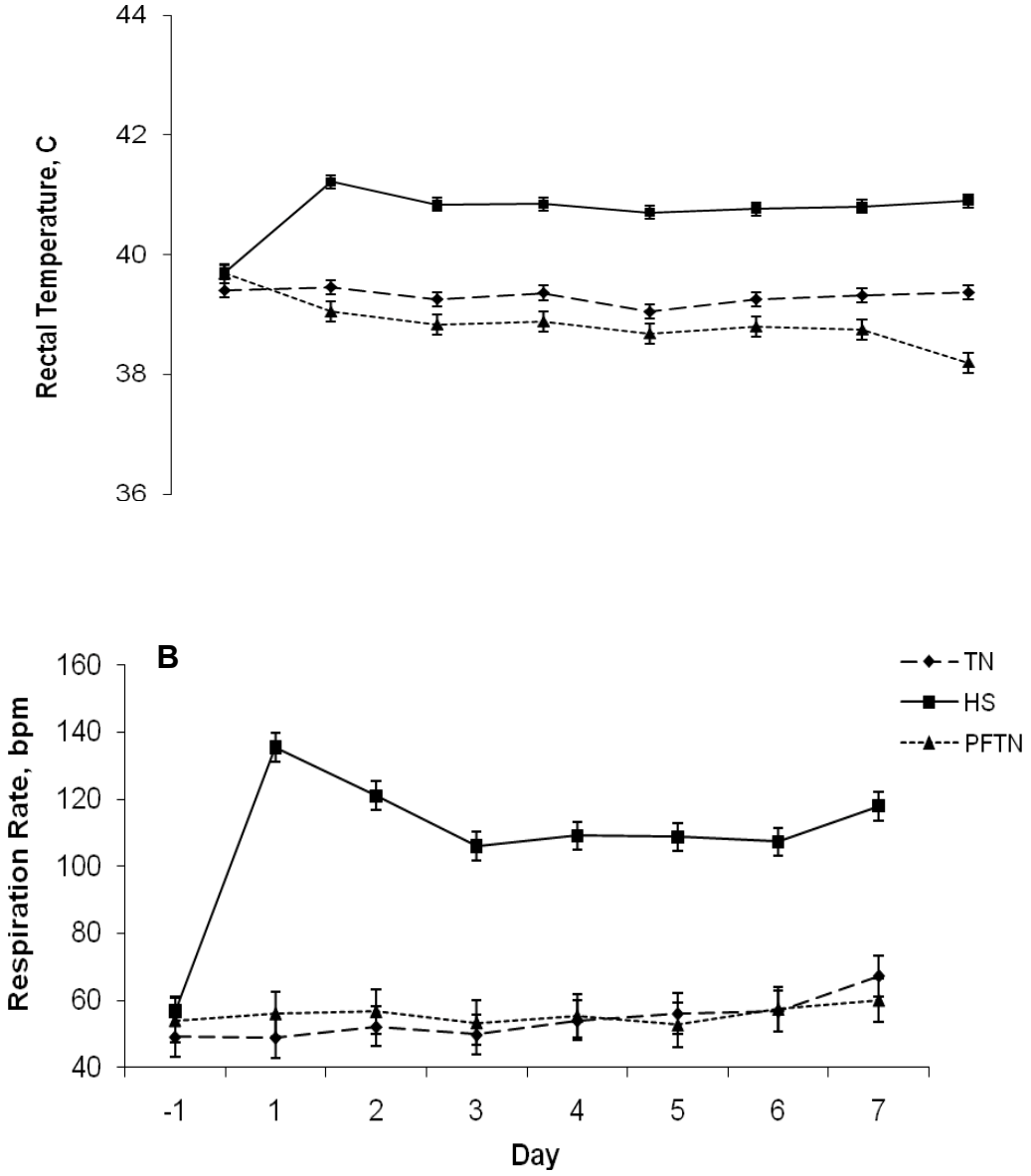

Figure 1: Effects of constant environment [ad-libitum intake in thermal neutral (TN; 20°C) conditions; heat stress (HS; 35°C) or pairfeeding in thermal neutral conditions (PFTN)] on (A) Rectal temperatures and (B) Respiration rates at 2000h in growing pigs.

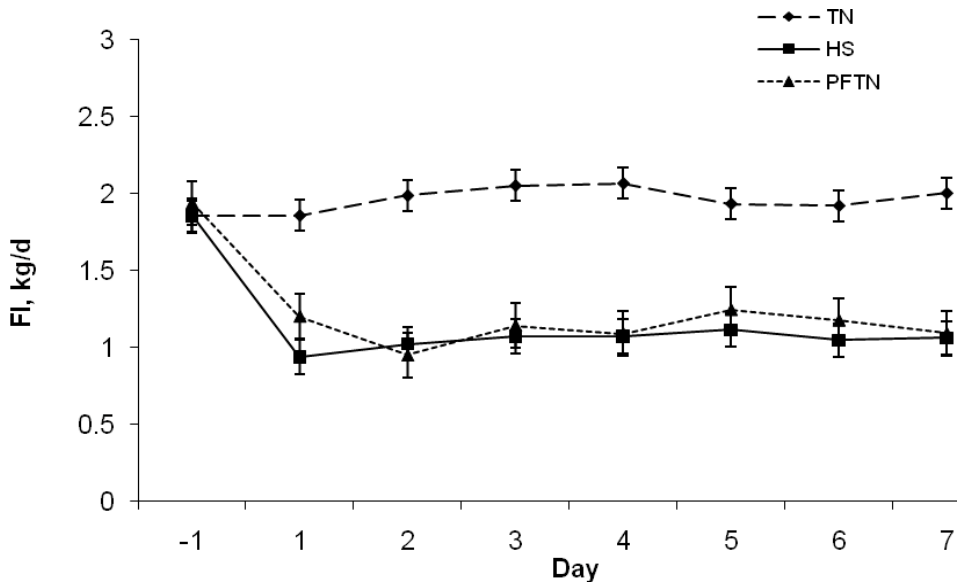

Figure 2: Effects of constant environment [ad-libitum intake in thermal neutral (TN; 20°C) conditions; heat stress (HS; 35°C) or pairfeeding in thermal neutral conditions (PFTN)] on daily feed intake in growing pigs.

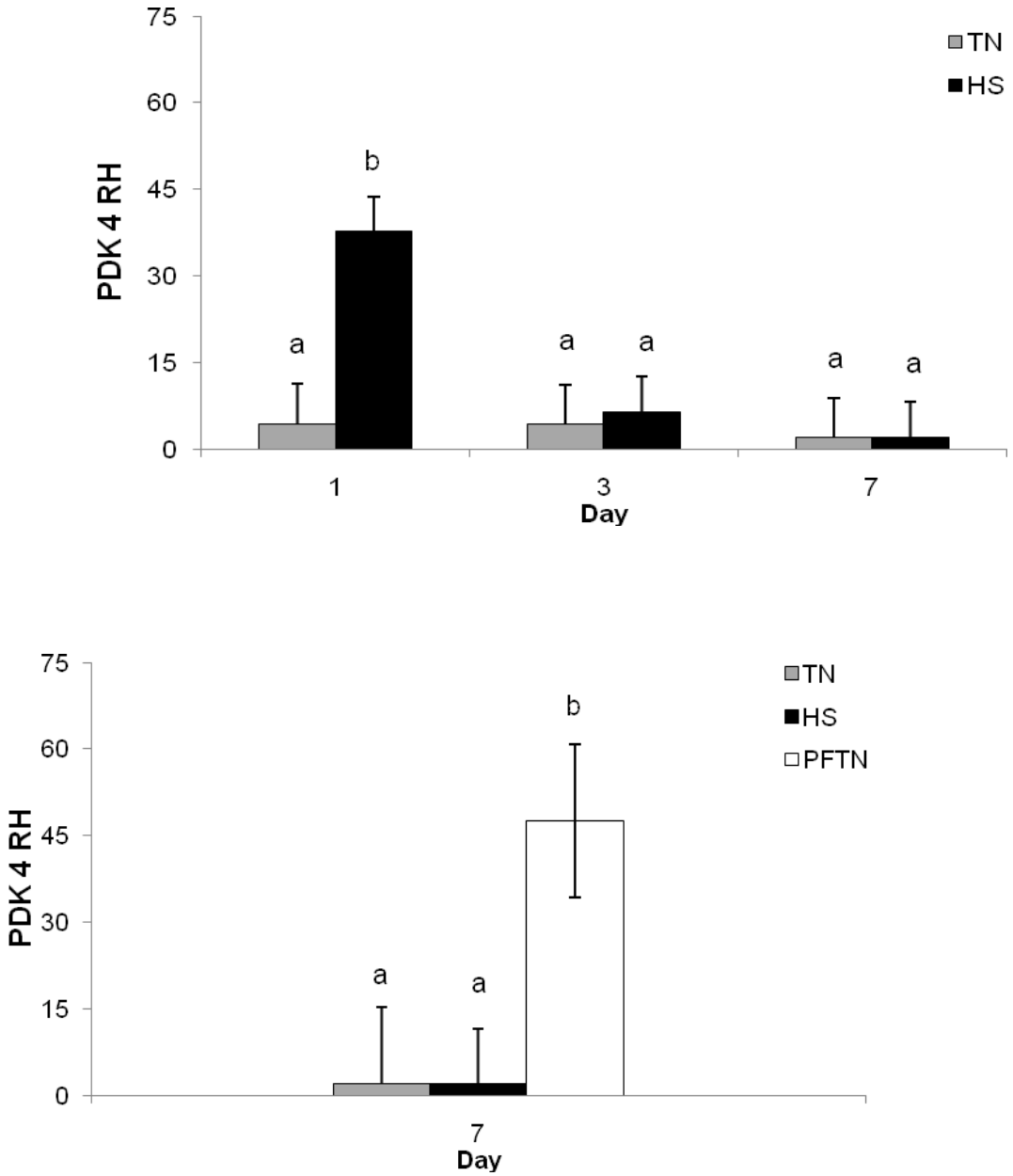

Figure 3: Effects of ad-libitum feed intake in thermal neutral conditions (TN; 20°C) and heat stress (HS; 35°C) conditions on the (A) temporal change in body weights and (B) effects of TN, HS, and pair-feeding in thermal neutral conditions (PFTN) on PDK4 gene expression at the end of the experiment. $^{a,b,c}P<0.05$

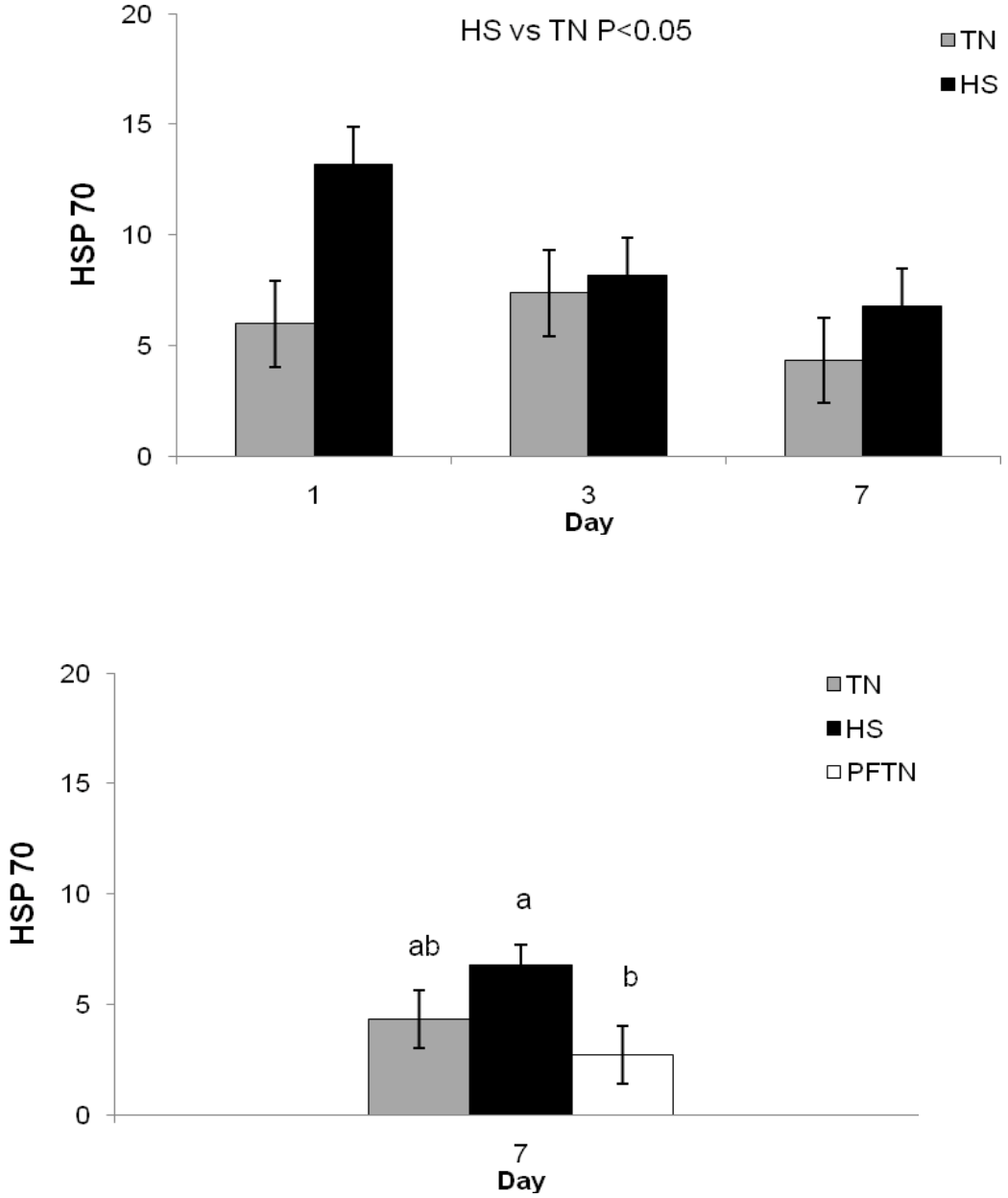

Figure 4: Effects of ad-libitum feed intake in thermal neutral conditions (TN; 20°C) and heat stress (HS; 35°C) conditions on the (A) temporal change in body weights and (B) effects of TN, HS, and pair-feeding in thermal neutral conditions (PFTN) on HSP70 gene expression at the end of the experiment. $^{a,b,c}P<0.05$

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