

Acute and chronic heat stress alters the metabolic profile of skeletal  
muscle in growing swine

Samantha Gwai Lan Won

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University  
in partial fulfillment of the requirements for the degree of

Master of Science  
In  
Animal and Poultry Sciences

Robert P. Rhoads  
Benjamin A. Corl  
David E. Gerrard  
Honglin Jiang

August 2, 2012  
Blacksburg, VA

Keywords: Heat stress, swine, metabolism, mTOR

# Acute and chronic heat stress alters the metabolic profile of skeletal muscle in growing swine

Samantha Gwai Lan Won

## ABSTRACT

Heat stress (HS) causes significant losses to the U.S. swine industry in several production and health areas including efficient lean tissue accretion. Perturbations in skeletal muscle metabolism may participate in this defect. The study objectives were to examine the cellular bioenergetic profile in skeletal muscle of piglets subjected to thermal stress in utero and/or during postnatal life. To accomplish this, 96 offspring from 14 sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or HS conditions (cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. At 14 weeks of age, offspring were exposed to one of two postnatal thermal environments, constant TN (21°C) or HS (35°C) for 24 hrs (acute study) or 5 weeks (chronic study). Pigs were sacrificed after treatment and longissimus dorsi skeletal muscle samples collected for molecular analyses. Differences ( $p < 0.05$ ) were observed in protein abundance of p-4eBP1 and total Rs6 and gene expression of Cox5B, CytB, EEF2, HK2, MURF, ND1, PGC-1 $\alpha$ , SDHA, and TFAM during the acute heat stress study. Differences ( $p < 0.05$ ) were observed in protein abundance of 4eBP1, total Akt, and p-Rs6 and gene expression of CytB, MURF, and PGC-1 $\alpha$  during the chronic heat stress study. These data indicate that acute postnatal HS alters skeletal muscle metabolism, which may favor a reduction in mitochondrial respiration and protein synthesis potentially via the mTOR pathway.

Keywords: Swine; Heat Stress; Metabolism; mTOR

## ACKNOWLEDGEMENTS

I would like to express my deepest appreciation and gratitude to Dr. Robert Rhoads for the opportunity to join his lab so far along in the pursuit of my degree. I would like to thank him over and over for his experienced guidance, his honest evaluation of my performance, and his willingness to be physically there whenever I needed assistance. He is one of the best things that has ever happened to me.

I would also like to express my most sincere gratitude to the other members of my committee, Drs. Honglin Jiang, Dave Gerrard, and Benjamin Corl for their patience and flexibility throughout the course of my stay here. It has been a crazy journey.

Very special thanks go to Guohao Xie for assisting me in the lab when things got difficult, and for taking the time to help me to get comfortable with the techniques that I needed to learn. He has become a wonderful mentor and friend.

I would also like to thank Lee Johnson, Britni Brown, Xiaomei Ge, Lidan Zhao, and Aihua Wang specifically for their constant encouragement and support throughout my two years.

I sincerely appreciate the support and encouragement of the faculty, staff, and other graduate students in Animal and Poultry Sciences. I would not have made it through without all of you.

Finally, I would like to thank my family, Delmond, Deanna, and Trevor Won as well as my boyfriend, Sanjay Giridhar, and my cheerleaders, Amanda Sebastian, Megan Lange, and my Hill and Veil family. They have provided countless hours of support, encouragement, and coffee when I most needed it.

## Table of Contents

<b>Abstract</b>	
<b>Acknowledgements</b> .....	iii
<b>Table of Contents</b> .....	iv
<b>List of Abbreviations</b> .....	v
<b>List of Tables</b> .....	vii
<b>List of Figures</b> .....	viii
 <b>CHAPTER I. Review of Literature</b>	
Heat stress and the swine industry .....	1
Heat stress effects on pigs.....	3
Carbohydrate metabolism .....	6
Mammalian target of rapamycin (mTOR) pathway and signaling .....	10
Heat shock proteins.....	13
Heat stress effects on metabolism .....	14
Heat stress effects on muscle through mTOR.....	18
 <b>CHAPTER II. Effects of acute and chronic heat stress on the mTOR pathway in swine</b>	
Abstract.....	21
Introduction.....	22
Materials and Methods.....	23
Results.....	27
Discussion.....	29
 <b>CHAPTER III. Effects of acute and chronic heat stress on metabolic gene expression in swine</b>	
Abstract.....	52
Introduction.....	53
Materials and Methods.....	54
Results.....	58
Discussion.....	61
<b>Literature Cited</b> .....	99
<b>Appendix A</b> .....	106

## **List of Abbreviations**

- 4eBP1 – Eukaryotic translation initiation factor 4e-binding protein 1
- ADG – Average daily gain
- Akt – Protein kinase B (p-Akt indicates the phosphorylated Akt)
- AMPK – AMP-activated protein kinase
- ATR – Atrogin-1
- Cox5B – Cytochrome C oxidase subunit B
- CytB – Cytochrome B
- DMI – Dry matter intake
- EEF2 – Elongation factor 2 (p-EEF2 indicates the phosphorylated EEF2)
- eIF2 – Eukaryotic initiation factor 2
- GAPDH – Glyceraldehyde 3-phosphate dehydrogenase
- HK2 – Hexokinase 2
- HS – Postnatal heat stress treatment
- HSHS – Gestational treatment (heat stress conditions throughout gestation)
- HSP70 – Heat shock protein 70
- HSTN – Gestational treatment (heat stress conditions through first half gestation)
- mTOR – Mammalian target of rapamycin
- MURF – Muscle ring finger protein 2
- ND1 – NADH dehydrogenase, subunit 1
- PDC – Pyruvate dehydrogenase complex
- PDH – Pyruvate dehydrogenase (p-PDH indicates the phosphorylated PDH)
- PDK4 – Pyruvate dehydrogenase kinase isoenzyme 4

PGC-1 $\alpha$  – Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PKM2 – Pyruvate kinase isoenzyme 2

PPAR $\gamma$  – Peroxisome proliferator activated receptor gamma

RH – Relative humidity

RNA – Ribonucleic acid

RR – Respiratory rate

Rs6 – Ribosomal S6 kinase (p-Rs6 indicates the phosphorylated Rs6)

SDHA – Succinate dehydrogenase complex, subunit A, flavoprotein variant

TFAM – Transcription factor A, mitochondrial

TN – Postnatal thermoneutral treatment

TNHS – Gestational treatment (heat stress conditions through second half gestation)

TNTN – Gestational treatment (thermoneutral conditions throughout gestation)

VFI – Voluntary feed intake

## List of Tables

3.1 Primers used in RT-PCR for skeletal muscle samples.....	65
---	----

## List of Figures

1.1 The mTOR complex and its associated regulators .....	12
2.1 Effects of acute heat stress on abundance of total 4eBP1 in skeletal muscle .....	32
2.2 Effects of sex on abundance of 4eBP1 in swine under acute heat stress .....	33
2.3 Effects of acute heat stress on abundance of phosphorylated 4eBP1 in skeletal muscle .....	34
2.4 Effects of acute heat stress on the ratio of the abundance of phosphorylated 4eBP1 to total 4eBP1 in skeletal muscle .....	35
2.5 Effects of acute heat stress on abundance of total Akt in skeletal muscle.....	36
2.6 Effects of acute heat stress on abundance of phosphorylated Akt in skeletal muscle .....	37
2.7 Effects of acute heat stress on the ratio of the abundance of phosphorylated Akt to total Akt in skeletal muscle.....	38
2.8 Effects of acute heat stress on abundance of total EEf2 in skeletal muscle.....	39
2.9 Effects of acute heat stress on abundance of phosphorylated EEf2 in skeletal muscle .....	40
2.10 Effects of acute heat stress on abundance of total Rs6 in skeletal muscle .....	41
2.11 Effects of acute heat stress on abundance of phosphorylated Rs6 in skeletal muscle .....	42
2.12 Effects of chronic heat stress on abundance of total 4eBP1 in skeletal muscle .....	43
2.13 Effects of chronic heat stress on abundance of phosphorylated 4eBP1 in skeletal muscle .....	44
2.14 Effects of chronic heat stress on abundance of total Akt in skeletal muscle .....	45

2.15 Effects of chronic heat stress on abundance of phosphorylated Akt in skeletal muscle .....	46
2.16 Effects of sex on abundance of phosphorylated Akt in swine under chronic heat stress.....	47
2.17 Effects of chronic heat stress on abundance of total EEF2 in skeletal muscle .....	48
2.18 Effects of chronic heat stress on abundance of phosphorylated EEF2 in skeletal muscle .....	49
2.19 Effects of chronic heat stress on abundance of total Rs6 in skeletal muscle .....	50
2.20 Effects of chronic heat stress on abundance of phosphorylated Rs6 in skeletal muscle .....	51
3.1 Effects of acute heat stress on abundance of total GAPDH in skeletal muscle .....	66
3.2 Effects of acute heat stress on abundance of total PDH in skeletal muscle.....	67
3.3 Effects of acute heat stress on abundance of phosphorylated PDH in skeletal muscle .....	68
3.4 Effects of acute heat stress on abundance of total PKM2 in skeletal muscle .....	69
3.5 Effects of chronic heat stress on abundance of total GAPDH in skeletal muscle .....	70
3.6 Effects of chronic heat stress on abundance of total PDH in skeletal muscle .....	71
3.7 Effects of chronic heat stress on abundance of phosphorylated PDH in skeletal muscle .....	72
3.8 Effects of chronic heat stress on abundance of total PKM2 in skeletal muscle .....	73
3.9 Effects of acute heat stress on ATR gene expression in skeletal muscle.....	74
3.10 Effects of acute heat stress on Cox5B gene expression in skeletal muscle .....	75
3.11 Effects of acute heat stress on CytB gene expression in skeletal muscle .....	76

3.12 Effects of acute heat stress on EEF2 gene expression in skeletal muscle .....	77
3.13 Effects of acute heat stress on HK2 gene expression in skeletal muscle.....	78
3.14 Effects of sex on HK2 gene expression in swine under acute heat stress.....	79
3.15 Effects of acute heat stress on HSP70 gene expression in skeletal muscle .....	80
3.16 Effects of acute heat stress on MURF gene expression in skeletal muscle .....	81
3.17 Effects of sex on MURF gene expression in swine under acute heat stress .....	82
3.18 Effects of acute heat stress on ND1 gene expression in skeletal muscle.....	83
3.19 Effects of acute heat stress on PDK4 gene expression in skeletal muscle.....	84
3.20 Effects of acute heat stress on PGC-1 $\alpha$ gene expression in skeletal muscle .....	85
3.21 Effects of acute heat stress on SDHA gene expression in skeletal muscle.....	86
3.22 Effects of acute heat stress on TFAM gene expression in skeletal muscle .....	87
3.23 Effects of chronic heat stress on ATR gene expression in skeletal muscle .....	88
3.24 Effects of chronic heat stress on Cox5B gene expression in skeletal muscle.....	89
3.25 Effects of chronic heat stress on CytB gene expression in skeletal muscle.....	90
3.26 Effects of chronic heat stress on HK2 gene expression in skeletal muscle .....	91
3.27 Effects of chronic heat stress on HSP70 gene expression in skeletal muscle.....	92
3.28 Effects of chronic heat stress on MURF gene expression in skeletal muscle.....	93
3.29 Effects of chronic heat stress on ND1 gene expression in skeletal muscle .....	94
3.30 Effects of chronic heat stress on PDK4 gene expression in skeletal muscle .....	95
3.31 Effects of chronic heat stress on PGC-1 $\alpha$ gene expression in skeletal muscle.....	96
3.32 Effects of chronic heat stress on SDHA gene expression in skeletal muscle .....	97
3.33 Effects of chronic heat stress on TFAM gene expression in skeletal muscle.....	98

# CHAPTER I

## REVIEW OF LITERATURE

### **Heat stress and the swine industry**

Heat stress is a major concern in the swine industry, not only in tropical areas where pigs are produced, but also in temperate areas such as the United States. Most swine production in the U.S. occurs in states that have hot summers and an increasing incidence of heat waves due to global climate change (Renaudeau et al., 2011). Millions of dollars each year are lost in the swine industry as a result of heat stress to the animals. Sow losses are estimated to be around \$113 million a year, while losses in the growing-finishing swine sector are estimated to be around \$202 million a year (St-Pierre et al., 2003). Economic losses in the swine industry come in many forms, and are dependent upon the sector in question.

The breeding sector of swine production is concerned with the ability of animals to breed, maintain healthy pregnancies, and bear litters within an optimal time period. Economic losses in the breeding sector stem from decreases in fertility of males and females, dry matter intake (DMI) and direct heat stress effects on maintenance of pregnancy. The fertility of boars has been found to decline for up to five weeks following the initiation of a heat stress event as measured through sperm output and semen quality. Decreased conception rates and litter sizes were also found in gilts exposed to heat stress between days 0 and 16 post-mating (Wettemann and Bazer, 1985). Heat stress is linked to decreased milk yield and increasing the energetic cost of lactation in sows, affecting the growth and survival of litters (St-Pierre et al., 2003). Lactating sows have a much higher energy requirement than sows that are not lactating. Decreased DMI in lactating sows

responding to heat stress causes weight loss, as the energy input is no longer sufficient for body maintenance and lactation. Sows that have been exposed to heat stress also take longer to return to estrus after weaning than sows that were not previously exposed to heat stress (Spencer et al., 2003). More days open between litters decrease a producer's profit, as fewer litters per year are weaned.

Although heat stress is thought to have less of a negative impact on growing-finishing pigs compared to reproductively active sows and boars, there is still a loss in growth rate as temperature increases. There is also a difference in energy partitioning between pigs that are heat stressed and pigs under thermoneutral conditions. Heat stress induces decreases in voluntary feed intake (VFI) in an attempt by the animal to produce less heat. This has direct effects on rate of growth as smaller quantities of feed are ingested over time (Le Bellego et al., 2002). Diet composition is also important in growing-finishing pigs under heat stress, as larger amounts of protein in feed require more energy to metabolize as measured through urea nitrogen concentration (Kerr et al., 2003). Energy is partitioned increasingly to fat as temperature increases, which is undesirable for a swine producer concerned with lean muscle deposition (Le Bellego et al., 2002). The negative effects of heat stress on growth are increased when the relative humidity (RH) increases. As RH increases, the upper critical temperature for pigs tends to decrease as they are unable to rely as heavily on evaporative heat loss through panting. In a study by Huynh et al. (2005), the greatest decrease in average daily gain (ADG) was found at a RH of 80%. However, these findings are most likely influenced by a combination of high temperature, RH, and decreased VFI rather than any independent factor.

## **Heat stress effects on pigs**

The impacts of heat stress on swine production are of particular interest in research due to the limited capacity of pigs to lose heat via evaporation of water from the skin. This affects the management practices taken to ensure that animals are protected and healthy, especially during hot and humid summer months. Pigs must rely on other mechanisms of heat dissipation as well as a reduction in body heat production. Some of the primary heat coping responses utilized in pigs under heat stress include panting, exposure of skin to cool surfaces, reduced activity, and decreased VFI.

The temperatures at which pigs are most likely to display behaviors and physiological changes based upon heat stress depend a great deal upon the body weight of the animal. As pigs grow, the ratio of surface area to mass decreases, and there is a higher amount of energy intake relative to maintenance energy levels leading to decreased heat dissipation efficiency (Renaudeau et al., 2011).

Panting is one of the first responses pigs utilize in an attempt to dissipate heat. Panting allows the animal to cool itself through exposing moist respiratory passages to outside air, and is the main form of evaporative cooling available in pigs (Fuquay, 1981). Huynh et al. (2005) estimate that panting begins to increase at around 22.4°C in pigs weighing approximately 60 kg. Respiratory rate (RR) was found to increase significantly in both stress resistant and stress susceptible pigs exposed to a heat stress of 35°C and 65% RH (Aberle et al., 1974). Panting is less effective for heat loss in more humid environments, since swine cannot add as much water to the air (Huynh et al., 2005). In terminally heat stressed pigs, increases in RR increased from the start of the trial through

40 minutes prior to death. A decline was observed for the last 40 minutes of the trial period (Marple et al., 1974).

In a study by Aarnink et al. (2006), pigs were shown to lie on their sides with greater frequency (0.5% more pigs per °C) as temperature increased, exposing more body surface area to the cooler floors. Pigs were also less likely to lie against other animals, thus avoiding other potential sources of heating. The temperature at which pigs were likely to begin lying on their sides decreased as body weight increased, again emphasizing that higher body weights affect the efficiency of heat dissipation. Lying is also a way to decrease the activity level of the animal, which subsequently decreases heat production from movement. Wallowing, a behavior performed by swine at all temperatures has also been found to increase as temperature rises (Huynh et al., 2005). Wallowing is characterized as rolling in urine and fecal matter, and is used for evaporative heat loss from the skin of swine. Total heat production in piglets was reported to decrease by 20% with an increase in temperature from 23°C to 33°C. This was due to a decrease in activity level. Feeding level had no effect on this decrease as piglets were fed ad libitum (Collin et al., 2001).

There is a great deal of literature exploring the effects of heat stress on feed intake in swine. As temperature increases, VFI decreases so that the animal may produce less heat from energy metabolism. This is especially important since today's animals have been selected for a high rate of metabolism for quick growth and as a result, produce more heat (Renaudeau et al., 2008). Collin et al. (2001) reported a 25% decrease in VFI for piglets at 33°C when compared to piglets at 23°C. A different study reported a decrease of 95.5 g for every increase in °C above the upper critical temperature (Huynh,

et al., 2005). In a diurnal heat stress study, a significant time of day and temperature interaction on VFI was found. Heat stressed swine consumed larger quantities of feed in the morning when temperatures were cooler compared to the evening in which temperature increased (Patience et al., 2005). Decreases in values for VFI over time are dependent upon the study, since there is a large amount of variation in experimental design and animals. However, there is consensus that heat stress causes overall decreases in VFI, and that the effect depends upon the level of heat stress, the composition of the diet, and the body weight of the animals.

Through a combination of decreases in VFI and activity level, swine are able to decrease their maintenance energy requirement. The maintenance energy requirements for 9 to 20 kg swine compared between 14°C and 32°C were 4.6 MJ DE per day and 2.18 MJ DE per day respectively (Campbell and Taverner, 1988).

If left untreated, heat stress in swine can cause serious health issues. Death may occur if the animal is no longer able to dissipate heat successfully, indicated by increases in internal body temperature. These increases indicate that the animal is no longer able to maintain its internal temperature homeostasis. In terminally heat stressed swine, death occurred at a rectal temperature of 43°C (Marple et al., 1974). However, rectal temperature should not be used as the first indication of heat stress in swine. Increases in rectal temperature have been reported to occur at temperatures of 3-4°C above that at which RR begins to increase. Thus, monitoring of RR would yield much quicker information about an animal's comfort level before significant physiological damage occurs (Huynh et al., 2005).

## **Carbohydrate metabolism**

Animals require an input of free energy to support the mechanical work of muscle contraction and cell movement, active transport of molecules and ions across cell membranes and the synthesis of macromolecules required by the cell (Himms-Hagen, 1976). Cellular energy requirements are met by the metabolism of substrates including glucose, lactate, fatty acids and amino acids, through a series of highly integrated chemical reactions. Substrates can be converted into a number of metabolic intermediates and used in synthesis reactions, or utilized for the production of adenosine triphosphate (ATP). The generation of ATP is a highly regulated process involving three distinct pathways generally termed glycolysis, the tricarboxylic acid cycle (TCA) and oxidative phosphorylation (via the electron transport chain). Pathway regulation is determined by several mechanisms affecting enzyme activity including synthesis and degradation rates, allosteric interactions and covalent modification and the energy charge of the cell (Campbell, 1999; Berg et al., 2007). The energy charge of the cell is determined by the relative amounts of ATP, ADP and AMP, where a high energy charge stimulates anabolic reactions and a low energy charge stimulates catabolism.

Carbohydrates are a readily available energy source that can be utilized in various ways in swine. Metabolism of carbohydrates involves a trade off between the glycolytic pathway and the tricarboxylic acid (TCA) cycle (Sugden and Holness, 2003). The primary function of glycolysis is to quickly generate 2 ATP molecules and 2 pyruvate molecules from glucose. The TCA cycle, by comparison, generates a much larger amount of ATP in a more efficient manner. The TCA cycle is dependent upon the presence of oxygen, and yields products that may be used in fatty acid synthesis.

There are three main rate limiting, essentially irreversible steps in the glycolytic pathway. The first rate limiting step for glycolysis involves the action of an enzyme called hexokinase (HK). HK is responsible for the phosphorylation of glucose to glucose-6-phosphate. The transcription of HK is upregulated by increases in circulating insulin, which stimulates glucose uptake into tissues (Printz et al., 1993). Glucose is then converted to glucose-6-phosphate, which is unable to leave the cell and can be used for glycolysis or glycogen synthesis. As glucose-6-phosphate levels increase within the cell, the action of HK is inhibited (Lueck and Fromm, 1974).

The second rate limiting step in the glycolytic pathway involves the phosphorylation of fructose-6-phosphate by phosphofructokinase. This results in the creation of fructose-1,6-bisphosphate, which can serve as an allosteric regulator of pyruvate kinase (the final rate limiting enzyme of glycolysis). Fructose-1,6-bisphosphate is committed to continuing through the glycolytic pathway. Prior to this reaction, glucose-6-phosphate can potentially bypass glycolysis and enter the pentose phosphate pathway. It can also be converted to glucose-1-phosphate and be used for glycogen synthesis.

After fructose-1,6-bisphosphate is formed, there are a series of less prominent reactions which ultimately result in the conversion of phosphoenolpyruvate to pyruvate. This final rate limiting step involves the transfer of a phosphorous from phosphoenolpyruvate to ADP to create ATP. Pyruvate kinase is the key enzyme that regulates this transfer. Increases in the activity of phosphofructokinase, and concurrent increases in fructose-1,6-bisphosphate also play a role in increasing the reaction rate of pyruvate kinase. At the end of the glycolytic pathway, there is a net yield of 2 ATP

molecules and 2 pyruvate molecules. The 2 pyruvate molecules from glycolysis may then enter the TCA cycle.

The enzymes that regulate the flow of pyruvate between glycolysis and the TCA cycle are collectively called the pyruvate dehydrogenase complex (PDC). The PDC is responsible for the conversion of pyruvate to acetyl-CoA, and the release of NADH for entry into the TCA cycle (Sugden and Holness, 2003). Increases in available pyruvate, and decreases in the amounts of acetyl-CoA and NADH increase the activity of the PDC.

The PDC is made up of three component enzymes that contribute to pyruvate decarboxylation. The major phosphorylating component, responsible for this irreversible transition is pyruvate dehydrogenase itself (E1). The other two components are dihydrolipoamide transferase (E2) and dihydrolipoamide dehydrogenase (E3).

Regulation of this activation and inactivation is important in the balance between glucose and fatty acid utilization (Sugden and Holness, 2006). There are two key enzyme families that play a role in the activation and inactivation of the PDC. These enzymes specifically affect the action of the E1 component.

The first family of enzymes includes the pyruvate dehydrogenase kinases (PDK). To date, there are four different isoforms of PDKs, which can be expressed in different frequencies depending on the tissue type (Gudi et al., 1995). PDKs are responsible for phosphorylation and inactivation of the PDC. Increases in fatty acids increase the activity of PDKs shifting the balance from glucose oxidation to fatty acid oxidation in order to conserve available glucose. However, this may also be due to decreases in insulin secretion, as insulin has been found to have a suppressive effect on PDKs in rat skeletal muscle (Lee et al., 2004). Regulation of PDKs is especially important in starvation states

with decreased substrate for glycolytic metabolism. During a five hour glucose deprivation, PDK mRNA levels were found to significantly increase in human myoblasts (Abbot et al., 2005). The preferred substrates then become fatty acids which have a higher energy yield, and have been previously stored in the body.

The second family of enzymes includes the pyruvate dehydrogenase phosphatases (PDP). PDPs are responsible for dephosphorylating and activating the PDC. Activation of the PDC results in an increase in glucose utilization through the glycolytic pathway (Sugden and Holness, 2003).

Following conversion by the PDC, acetyl-CoA enters the TCA cycle where it is converted to citrate. Citrate is a molecule that may proceed through the entire TCA cycle, or be utilized for fatty acid synthesis. Increasing levels of citrate provide negative feedback to phosphofructokinase, slowing the rate of glycolysis. As citrate proceeds through the TCA cycle, it is converted to isocitrate,  $\alpha$ -ketoglutarate, succinyl-CoA, and then succinate. Succinate may then enter the electron transport chain, which is part of the process of oxidative phosphorylation. This step is regulated by the action of succinate dehydrogenase complex, subunit A (SDHA). Oxidative phosphorylation involves a series of electron transfer reactions from electron donors to electron receivers. These reactions generate a large amount of ATP, making oxidative phosphorylation a more efficient energy utilization tool when compared to glycolysis. Cytochrome C oxidase is the terminal enzyme in the oxidative phosphorylation process, and is responsible for creating an electrochemical gradient across the mitochondrial membrane (the basis for the electron transport chain).

Succinate may also continue on in the TCA cycle. This results in the formation of oxaloacetate, which can then be converted back to citrate and recycled. This provides more NADH, a molecule that is important as a reducing agent in the process of oxidative phosphorylation. NADH is produced by many of the dehydrogenases within the cycle, and provides negative feedback for the PDC as well as for citrate synthase. The negative feedback provided by increasing NADH ensures that there is controlled entry of substrate to the TCA cycle. Excess substrate may then be stored in the form of glycogen through alternative pathways.

Peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) is one of the genes responsible for controlling the switch between glycolysis and fatty acid oxidation. Increases in the abundance of fatty acids cause translocation of PPAR $\gamma$  to the nucleus in order to signal transcription. Transcription of PPAR $\gamma$  regulated genes results in greater fatty acid uptake and formation. A coactivator known as PPAR $\gamma$  coactivator 1 alpha (PGC-1 $\alpha$ ) is also important in regulating the action of PPAR $\gamma$ . PGC-1 $\alpha$  activation increases mitochondrial biogenesis, and is sensitive to a number of different physiological stresses (Wenz et al., 2008).

### **Mammalian target of rapamycin (mTOR) pathway and signaling**

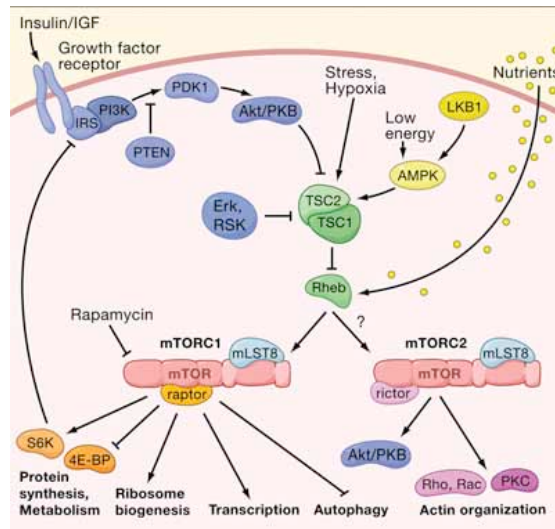
Mammalian target of rapamycin (mTOR) is a protein kinase of the phosphatidylinositol 3-kinase (PI3K) related kinase family that has recently been shown to be involved with the sensing of nutrient availability at the cellular level. Pathways involving mTOR regulate protein synthesis, and are sensitive to insulin signaling and physiological stress conditions (Sengupta et al., 2010). One signaling pathway that

appears to be strongly linked with mTOR is the PI3K/Akt pathway. PI3K signaling is activated through the binding of growth factors to receptors found in the cell membrane as well as through the binding of insulin to insulin receptors. Once activated, PI3K plays a role in the mediation of cell proliferation and survival (Polak and Hall, 2009).

Activation of PI3K through the insulin receptor pathway affects the activation of mTOR downstream. Increasing levels of nutrients cause the release of insulin, which is responsible for signaling the uptake of nutrients to the cell (Wullschleger et al., 2006). After insulin binds to its receptor, insulin tyrosine kinase is activated and phosphorylates insulin receptor substrates 1 and 2 (IRS 1/2). The IRS 1/2 can then be bound by PI3K, which will then convert PIP2 to PIP3. PIP3 goes on to activate Akt (also known as protein kinase B). Akt can then directly phosphorylate and activate mTOR.

Once activated, mTOR has two primary targets to act upon. The first is P70-S6-kinase. P70-S6-kinase is responsible for the activation of S6 ribosomal protein (Rs6). Once activated, Rs6 will initiate protein synthesis at the level of the ribosome. mTOR has been shown to phosphorylate P70-S6-kinase, which subsequently induces protein synthesis (Wullschleger et al., 2006). This may also have implications for cell proliferation. More specifically, studies show that P70-S6-kinase activation may increase the development and differentiation of adipose cells as well as growth and elongation of skeletal muscle.

The second major target of mTOR is eukaryotic translation initiation factor 4e-binding protein 1 (4eBP1; Sengupta et al., 2010). Under normal conditions, 4eBP1 is bound tightly to another protein called eukaryotic initiation factor 4e (eIF4E). eIF4E is inhibited by this interaction with the non-phosphorylated 4eBP1. Once 4eBP1 is



**Figure 1.1 The mTOR complex and its associated regulators (adapted from Wullschleger et al., 2006)**

phosphorylated by mTOR, eIF4E is released, allowing it to bind to mRNA (Wullschleger et al., 2006). It then recruits these mRNA to the ribosomal initiation complex for translation into proteins.

The ribosomal initiation factor complex is composed of a family of proteins called the eukaryotic initiation factors. Eukaryotic initiation factor 2 (eIF2) is also of interest when examining the regulation of protein synthesis at the ribosomal level, and is responsive to signaling through the Akt pathway. eIF2, like eIF4, is also involved with the process of translation and protein synthesis. However, eIF2 is primarily responsible for the initiation of translation rather than the recruitment of proteins to the ribosome. eIF2 binds tRNA to the ribosome through a GTP-dependent mechanism (Kimball, 1999). Under normal conditions, eIF2 remains phosphorylated. This state inhibits its ability to interact with its GTP exchange factor (eIF2B). Dephosphorylation allows eIF2 to dissociate from the initiation factor complex and to more easily exchange GDP for GTP. This exchange is responsible for the initiation of protein translation. For each cycle of protein translation, eIF2 must exchange GDP for GTP.

There is speculation about other potential targets for mTOR. For example, eukaryotic elongation factor 2 (EEF2), which is primarily responsible for the translation elongation step of protein synthesis (Sengupta et al., 2010). During this step, peptide bonds are formed between amino acids in order to create polypeptides. EEF2 is specifically involved with the translocation of peptides from the A-site (where tRNA is bound to an amino acid) to the P-site (where elongation of peptides occurs) of the ribosome. Overall, mTOR is proving to be an interesting player in the regulation of protein synthesis.

### **Heat shock proteins**

The cellular response to a heat load includes activation of transcription factors such as Heat Shock Factor 1 (HSF1; Morimoto, 1998), expression of proteins associated with acute homeostatic response such as Heat Shock Proteins (HSPs), and altered expression of molecules (i.e. transcription factors), that lead to changes in the expression of proteins necessary for restoring cellular function and directing cellular remodeling, such as regulatory proteins, cell-cycle control proteins and structural proteins, as well as those that determine cell fate, including proteins involved in pro- and anti-apoptotic pathways (Sonna et al., 2002).

Heat shock proteins are a family of proteins, upregulated by HSF1 during times where the temperature becomes highly elevated. Heat shock proteins are also upregulated in response to nutrient depletion, exercise, and inflammation (Kregel, 2002). They are responsible for regulating the folding and synthesis of proteins. Heat shock proteins may

act as chaperones during times of environmental stress in order to ensure the protection of proteins.

Increased heat shock protein expression has been associated with decreased muscle hypertrophy in rats (Frier and Locke, 2007). In this study, rats that were not exposed to heat stress displayed significant increases in muscle mass after muscle overload. These rats also exhibited an increase in HSP 25 and 72 following overload. Rats that were previously exposed to heat stress exhibited HSP expression that was higher overall than the HSP expression from overload alone. Heat stressed rats displayed lower muscle mass increases after overload than the rats that had not been heat stressed.

### **Heat stress effects on metabolism**

Heat stress can affect animal metabolism in multiple ways. The first simply stems from the fact that heat stress decreases the rate of feed consumption. Animals exposed to heat stress do not consume as much feed in order to avoid excess heat production through the process of digestion. This results in loss of body weight and declines in feed efficiency. In swine, it has been found that both the time spent feeding and the meal size was significantly decreased in response to heat stress conditions (Collin et al., 2001). Heat stressed broiler chickens have been found to exhibit sharp declines in weight gain and feed efficiency even when consuming the same amounts of feed (Geraert et al., 1996). Growing beef cattle have also been found to display decreases in feed intake that may be directly attributable to decreases in average daily gain (O'Brien et al., 2010). This implies that heat stress not only affects the amount of feed consumed, but also the way that nutrients are processed and absorbed.

Heat stress is responsible for changing the way in which energy is partitioned. In swine, heat stress decreases the rate of protein and lipid deposition (Campbell and Taverner, 1988). This is undesirable not only because of the subsequent decline in growth rate to market weight, but also because the partitioning of energy becomes skewed towards lipid deposition rather than protein deposition (Le Bellego et al., 2002). A similar response was observed in broiler chickens, with declines in the rates of protein deposition and protein retention, and increases in the retention of fat (Geraert et al., 1996). Organisms are composed of proteins and lipid molecules that are sensitive to temperature change outside a relatively narrow window. Heat stress in particular may denature proteins and cause extensive cellular damage depending on the length and intensity of the exposure. Significant heat loads have also been found to cause damage to skeletal muscle as evidenced by increasing circulatory levels of creatine kinase (Aberle et al., 1974). In humans, ammonia levels have been found to increase along with heat load during exercise conditions (Febbraio, 2001).

Despite extensive research in the area of heat stress, data remain obscure regarding metabolic and biochemical changes that occur during heat exposure. Metabolic adaptations to a heat load likely occur in order to increase survival probability. Lee and Scott (1916) hypothesized a shift in fuel supply to skeletal muscles is necessary to decrease metabolic heat production. Fink et al. (1975) were first to demonstrate the effect of environmental temperature on substrate utilization by skeletal muscle indicating enhanced intramuscular carbohydrate use when exercising in the heat compared to cooler environments. Similarly, prevention or reduction in heat load is associated with reduced muscle glycogenolytic rate and/or carbohydrate oxidation (Kozlowski et al., 1985;

Febbraio et al., 1994a, 1994b, 1996; Gonzalez-Alonso et al., 1999). Glucose disappearance did not differ between exercise at 40°C compared to 20°C, while estimates of carbohydrate utilization were enhanced suggesting that during exercise in hot environments glucose uptake does not increase, but rather intramuscular glycogen breakdown is accelerated (Hargreaves et al., 1996). Moreover, Gonzalez-Alonso and coworkers (1999) measured glucose uptake by atrial-venous (AV) difference during muscle contraction and found no differences despite an increased respiratory quotient. These data suggest glycogen utilization is enhanced during exercise in a hot environment, which may be due to increased/enhanced anaerobic metabolism. Muscle lactate accumulation is reported in humans (Young et al., 1985; Febbraio et al., 1994a, b; Hargreaves et al., 1996; Gonzalez-Alonzo et al., 1999; Parkin et al., 1999) and dogs (Kozlowski et al., 1985) during exercise in the heat. Rowell and coworkers (1968) reported enhanced hepatic glucose production in heat-stressed humans, an effect that could not be blunted by exogenous glucose supply (Febbraio et al., 1996, 2001; Angus et al., 2001). However, some believe that the metabolic changes that occur as a result of thermal insult lead to a depletion in energy reserves, possibly due to altered regulation of key metabolic points such as the pyruvate dehydrogenase complex (Streffer, 1988). Decreased ratios of lactate/pyruvate and NADH/NAD<sup>+</sup> have been reported (Streffer, 1988) perhaps due to reduced oxygen supply.

Heat stress may also be damaging through increases in the production of reactive oxygen species. The mitochondria produce small amounts of reactive oxygen species through oxidative phosphorylation under normal conditions. Normally, cells are able to cope with small amounts of oxidative stress, and may detoxify free radicals that are

released by the mitochondria. Problems arise when these free radicals are produced in large amounts. In this case, they may damage genetic material as well as proteins and lipids. For example, heat stress induced increases in the production of reactive oxygen species in broilers have been associated with significant weight loss (Mujahid et al., 2005). PGC-1 $\alpha$  has been shown to increase along with reactive oxygen species, perhaps as a way for the body to detoxify through the formation of new mitochondria. Evidence from the literature indicates that mitochondria may be affected directly by heat stress (Polla et al., 1996; Bornman et al., 1998; Davidson and Schiestl, 2001; Heise et al., 2003; Qian et al., 2004). Histological analysis of skeletal muscle in a rodent heat stroke model showed mitochondrial abnormalities, denoted as ragged-red fibers (RRFs) and electron-microscopic observations revealed an increased number and size as well as altered morphology of mitochondria (Hsu et al., 1995). Mitochondria in RRFs appear to aggregate within the subsarcolemmal space, possibly indicating an increased energy demand of the plasma membrane due to hyperthermia (Hsu et al., 1995). Similarly, heat-stressed cardiomyocytes exhibit swollen mitochondria with broken cristae and low matrix density, in addition to reduced ATP content in the myocardium (Qian et al., 1992; Song et al., 2000). Because mitochondria are a major source of energy production within the cell, damage to mitochondria can impair a cell's ability to compensate for the increased energy demands (Hubbard, 1990) imposed by environmental stresses, and may contribute to increased levels of oxidative stress.

## **Heat stress effects on muscle through mTOR**

Skeletal muscle is one of many tissues that are significantly affected by heat stress. Heat can negatively impact cell components directly, such as unfolding and subsequent aggregation of proteins (Lindquist, 1986; Caspani et al., 2004, Roti Roti, 2008). Protein synthesis appears to be particularly impaired by heat. For example, Mondovi and coworkers (1969) noted that DNA, RNA and protein synthesis were rapidly inhibited by heat treatment, with the reduction being greatest for protein synthesis followed by DNA and finally RNA synthesis (Mondovi et al., 1969; Henle and Leeper, 1979). Furthermore, reductions in protein synthesis following heat exposure can be rapid occurring within 10 min at 42°C in HeLa cells (McCormick and Penman, 1969). Similarly, Henle and Leeper (1979) report significant reductions in synthesis rates for all macromolecules due to heat exposure, but note that despite protein synthesis being effected most, recovery was the fastest; resumption of DNA synthesis required a much longer period of time.

Heat stress may adversely affect metabolism and protein accretion via altered signaling through mTOR and its related proteins. This may be associated with concomitant declines in feed consumption, circulating nutrients and insulin. An absence of insulin signaling through the Akt pathway would lead to a decline in the activation of mTOR and protein synthesis. Studies have shown that Akt signaling plays a role in skeletal muscle hypertrophy. Akt is not only activated through insulin, but also through growth factors such as IGF-1 (Glass, 2003). With decreases in the amount of signaling through the Akt pathway, it is possible that protein deposition decreases through the inactivation of mTOR and its associated proteins located downstream.

An indirect way in which heat stress may affect mTOR is through AMP-activated protein kinase (AMPK) signaling. AMPK is an enzyme that plays an important role in energy homeostasis. Specifically, it is able to sense the ratio of AMP:ATP within the cell (Hood, 2009). High amounts of AMP signal that there is energy depletion. AMPK then inhibits pathways that would consume energy (cell growth) in favor of pathways that would conserve energy (fatty acid synthesis, glucose uptake). Since heat stress causes declines in the animal's feed consumption as well as decreases in nutrient uptake, it creates an environment in which there is less energy and more inhibition of cell growth through the AMPK pathway.

Proteins associated with, and downstream of the mTOR complex are also associated with muscle synthesis through their nutrient signaling functions. For example, Rs6 and 4eBP1 are both downstream proteins that are under the direct influence of mTOR (Wullscheleger et al., 2006). Rs6 functions as an initiator of ribosomal protein synthesis. 4eBP1, when phosphorylated, will release eIF2, which initiates the translation process of protein synthesis. Both of these processes may result in decreased protein deposition and muscle growth.

## **Summary**

The mTOR complex is known to be a regulator of protein synthesis in response to nutrient availability. Because heat stress conditions are known to skew nutrient partitioning from lean tissue accretion towards lipid deposition despite a reduction in the feed intake of the animal, it is likely that mTOR may be affected by heat stress. Indeed, activation of important downstream proteins, such as Rs6, appears to be altered by HS

conditions. Coupled with reported HS-related changes in cellular protein synthesis rates, the mTOR pathway is an attractive point of regulation. Therefore, the objective of the first experimental chapter is a first effort to examine the intermediate role of the mTOR complex between nutrient availability and protein synthesis and its associated key pathway regulation points during HS.

Cellular energy status (i.e. ATP/AMP ratio) is one of the primary mechanisms for determining substrate utilization. The literature points to alterations in cellular energy derivation from fuel substrates during HS with carbohydrate apparently favored over lipid. Moreover, studies point to increased anaerobic glycolysis in peripheral tissues and the Cori cycle may be a key mechanism to maintain glucose and energy homeostasis. Reduced oxidative glucose metabolism during HS may result from events decreasing oxidative phosphorylation and mitochondrial function. From a growth perspective, this strategy may limit energy necessary for anabolic processes and may explain why efficiency is compromised during elevated environmental temperatures. The objective of the second experimental chapter is to understand the cellular bioenergetic response to HS by measuring expression of genes involved in three key cellular metabolic pathways; glycolysis, the TCA cycle and oxidative phosphorylation.

## CHAPTER II

### Effects of acute and chronic heat stress on the mTOR pathway in swine

#### ABSTRACT

Heat stress is known to have detrimental effects on nutrient partitioning and protein accretion across livestock species. Pigs may be particularly susceptible due to their inability to cope efficiently with heat dissipation. Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or HS conditions (cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were divided into two constant TN (21°C) or HS (35°C) treatment groups (n=48 per treatment) at 14 weeks of age for the acute heat stress study (duration of 24 hours) and the chronic heat stress study (duration of 5 weeks). Skeletal muscle samples (longissimus dorsi) were collected at the end of each treatment period. Western blots were used in order to assess protein abundance within skeletal muscle. Significant decreases ( $p < 0.05$ ) were observed in abundance of p-4eBP1 and total Rs6 based on postnatal heat treatment, and in abundance of total Rs6 based on gestation treatment during the acute heat stress trial. A significant decrease ( $p < 0.05$ ) in the ratio of p-4eBP1:total 4eBP1 was also observed. Significant increases ( $p < 0.05$ ) were observed in abundance of total 4eBP1 and p-Rs6 based on gestational treatment during the chronic heat stress trial. These data suggest that there may be both immediate and prolonged effects of heat stress on protein synthesis at the molecular level in swine.

## Introduction

Climate change and the potential for heat stress remains a concern of high priority for swine producers in the United States (Reneaudeau et al., 2011). Pigs have a limited capacity for heat dissipation through evaporative cooling of the skin, and are therefore more susceptible to heat stress effects than other species. Pigs under heat stress conditions have been found to exhibit a variety of signs that are unfavorable to the swine producer. Examples include a lower voluntary feed intake (VFI; Collin et al., 2001), increased ratio of fat to protein deposited (Le Bellego et al., 2002), and decreased metabolic rate (Collin et al., 2001). All of these affect the product and time to produce animals for market.

Previous data show that offspring from sows exposed to heat stress conditions during the first half of gestation displayed increased back fat depth compared to all other gestational treatment groups. An interaction between prenatal heat stress and postnatal heat stress was also observed. Animals that had been exposed to heat stress prenatally were found to have decreased loin eye area after postnatal heat stress even though feed intake and growth rate were not significantly different (Boddicker et al., 2012). This implies that heat stress could potentially be altering metabolism in regards to the nutrient deposition of protein and fat. These data are consistent with other studies across species suggesting that there is a decrease in protein accretion under heat stress conditions, and an increase in lipid deposition (Geraert et al., 1996, Collin et al., 2001).

In an *in vitro* study by Kamanga-Sollo et al. (2011), porcine muscle satellite cells were examined in order to determine the effect of heat stress on skeletal muscle protein accretion. The results indicated that heat stress may actually increase protein synthesis

and decrease degradation. These data are surprising, as other studies have shown that protein synthesis decreases with increases in heat load (Collin et al., 2001). Conflicts between study results create the need for work examining the effects of heat stress on skeletal muscle in vivo.

This study examines the in vivo effects of heat stress on protein expression at the molecular level, and specifically focuses on the mTOR pathway. It seeks to answer the question: In what ways does heat stress affect skeletal muscle metabolism in growing swine? The mTOR pathway is a key intermediate in protein synthesis (Wullschleger et al., 2006), and could provide clues as to the mechanism behind heat stress effects on skeletal muscle metabolism and nutrient partitioning (Sengupta et al., 2010). Downstream initiators of protein synthesis as well as enzymes involved with cell survival and cell nutrient intake and signaling were also examined.

## **Materials and Methods**

### ***Animals and experimental design***

Animal gestational experiments were performed at the University of Missouri. A total of 15 sows were exposed prenatally to one of four gestational treatments. TNTN sows were exposed to thermoneutral conditions (TN, 25°C) for the entirety of their gestation period. HSHS sows were exposed to cyclical heat stress conditions (28-34°C) for all of gestation. HSTN sows were exposed to heat stress for only the first half of gestation. TNHS sows were exposed to heat stress for only the second half of gestation.

96 offspring from the four gestational treatment groups were transported to Iowa State University where they were placed into TN and HS treatment groups for postnatal heat treatments at 14 weeks of age. Animals used for the acute heat stress study (n=48) were exposed to either constant TN (21°C) or HS (35°C) conditions for 24 hours. Animals were sacrificed at the end of the study, and longissimus dorsi muscle samples were flash frozen in liquid nitrogen. Animals used for the chronic heat stress study (n=48) were exposed to either constant TN (21°C) or HS (35°C) conditions for five weeks. Animals were sacrificed at the end of the study, and longissimus dorsi muscle samples were flash frozen in liquid nitrogen.

### ***Protein extraction***

Longissimus dorsi (LD) muscle samples, collected in Iowa and shipped to Virginia Tech were transferred from a freezer set at -80°C to liquid nitrogen. Samples were kept frozen and pulverized in the original foil package, transferred to new Whirl-Pack bags, and placed back into liquid nitrogen for extraction. Approximately 100 mg of sample were transferred to a 5 mL plastic centrifuge tube containing 2 mL of cell lysis buffer (Cell Signaling). Tissue was homogenized in the lysis reagent using a Power Gen 125 homogenizer from Fisher Scientific set at 6 speed for two passes of approximately 15 seconds each. The homogenizer was cleaned with deionized water, and primed with lysis reagent between each sample. Samples were spun in a centrifuge set for 4°C at 8000 x g for 20 minutes. The supernatant was pipetted to a 2 mL snap cap tube and centrifuged at 4°C for 10 minutes at 12000 x g. The supernatant was used to create aliquots of

approximately 500  $\mu\text{L}$  each. Aliquots were stored at  $-80^{\circ}\text{C}$  for protein assay and Western blotting.

### ***BCA protein assay***

The Thermo Scientific Pierce Microplate BCA Protein Assay Kit was utilized to determine protein concentration values for each sample. Standards came from the Pre-Diluted Protein Assay Standards: Bovine Serum Albumin Set from Thermo Scientific. 22.5  $\mu\text{L}$  of autoclaved water and 2.5  $\mu\text{L}$  of each sample were pipetted to each well. Samples were pipetted in triplicate with 24 samples on each plate. BCA Protein Assay Reagents A and B were mixed in a 50:1 ratio. 200  $\mu\text{L}$  of the solution was then pipetted to each well. Plates were incubated for 30 minutes at  $37^{\circ}\text{C}$  and read using a Bio-Tek reader and KC Junior software.

### ***Western blot***

Protein samples were thawed from the  $-80^{\circ}\text{C}$  freezer on ice. Samples were diluted to a final concentration of 50  $\mu\text{g}$  protein using pure autoclaved water to a total volume of 35  $\mu\text{L}$ . A master mix was created, which included 12.5  $\mu\text{L}$  of sample buffer and 2.5  $\mu\text{L}$  of reducing agent per sample. 15  $\mu\text{L}$  of the master mix was added to each sample resulting in a total volume of 50  $\mu\text{L}$ . Samples were mixed, boiled for 5 minutes, and spun prior to gel loading. 10% and 12% Bis-Tris gels, and 8% Tris-Acetate gels (Bio-Rad) were used depending on the molecular weight of the protein of interest. Running buffers (Bio-Rad) were diluted and added to each chamber. 15  $\mu\text{L}$  of each sample were added to wells 2-25 of each gel. 1  $\mu\text{L}$  of ladder was added to well 1 of each gel. Gels ran for approximately

one hour at 200 V (10% and 12%) or 150 V (8% Tris-Acetate), until the sample buffer had completely dissipated into the running buffer. Gels were removed and transferred to Midi format membranes for 30 minutes using the Trans-Blot system by Bio-Rad. Following transfer, membranes were incubated for one hour in 20 mL PBS blocking buffer with constant shaking. Membranes were then transferred to sealed plastic bags and incubated with primary antibody (4e-BP1, p-4eBP1, Akt, p-Akt, EEF2, p-EEF2, Rs6, p-Rs6 from Cell Signaling) in 10 mL of PBS blocking buffer overnight at 4°C with constant shaking. After incubation, membranes were washed 5 times with PBS and 0.1% Tween detergent for 5 minutes each. Secondary antibody (IRDye Anti-rabbit from Licor) was then added to each membrane in the concentration of 1:20000 in PBS blocking buffer. Incubation with the secondary antibody was set for one hour with constant shaking. Following incubation, membranes were washed 3 times with PBS and 0.1% Tween detergent for 5 minutes each. Membranes were read for fluorescence using Odyssey software, version 3.0 from Li-Cor.

### ***Data analysis***

All data were analyzed using the proc mixed procedure of SAS. Analysis for protein abundance was based off of the equation:  $Y_{ijklm} = \mu + \alpha_i + \beta_j + \gamma_k + (\beta\gamma)_{jk} + \delta_{l(j)} + \zeta_m + \varepsilon_{ijklmn}$ , where  $\mu$  represents the overall mean of the population,  $\alpha_i$  represents the mean effect of sex,  $\beta_j$  represents the mean effect of gestation treatment,  $\gamma_k$  represents the mean effect of heat treatment,  $(\beta\gamma)_{jk}$  represents the interaction of gestation and heat treatments,  $\delta_{l(j)}$  represents the random effect of sow,  $\zeta_m$  represents the random effect of blot, and  $\varepsilon_{ijklmn}$

represents the unexplained residual element assumed to be independent and normally distributed. Results were considered to be significant with a p-value less than 0.05.

## Results

### *Acute heat stress results*

Protein expression of total and phosphorylated Akt did not change significantly during acute heat stress (Figures 2.4, 2.5), although there was a tendency for the ratio of p-Akt:total Akt to increase during acute heat stress (Figure 2.7). Proteins downstream of Akt seemed to be affected by heat stress, despite the small window of heat exposure. Specifically, proteins linked with the action of mTOR were shown to significantly decrease. Total Rs6 significantly decreased under acute heat stress conditions (Figure 2.8). This was more evident in pigs that were exposed to late gestation heat stress in utero ( $p < 0.05$ ). However, decreases were seen across the board in pigs that had been exposed to heat stress when compared to pigs that had been kept in thermoneutral conditions ( $p < 0.05$ ). While total Rs6 decreased, phosphorylated Rs6 remained unaffected by acute heat stress suggesting that the overall activity of this protein may have remained the same (Figure 2.9). The ratio of p-Rs6:total Rs6 was not significantly different (data not shown). A second downstream protein, 4eBP1, did not significantly change during acute heat stress (Figure 2.1). However, the active phosphorylated form of 4eBP1 was shown to decrease significantly (Figure 2.3;  $p < 0.05$ ). A significant decrease was also observed in the ratio of p-4eBP1:total 4eBP1 (Figure 2.4). This would suggest a concurrent decrease in translation of proteins. No significant differences were observed in levels of total or

phosphorylated EEF2 during the acute heat stress trial (Figures 2.6, 2.7). There was no significant difference observed in the ratio of p-EEF2:total EEF2 (data not shown).

### ***Chronic heat stress results***

When exposed to chronic heat stress, a significant increase in total Akt was observed in pigs that had been exposed to late gestation heat stress (Figure 2.12;  $p < 0.05$ ). However, there was no significant difference in phosphorylated Akt (Figure 2.13). There was no significant difference observed for the ratio of p-Akt:total Akt (data not shown). Downstream, 4eBP1 abundance was observed to significantly increase with late gestation heat stress exposure (Figure 2.10;  $p < 0.05$ ). Similarly to Akt, the active phosphorylated 4eBP1 did not change significantly (Figure 2.11). There was no significant difference observed for the ratio of p-4eBP1:total 4eBP1 (data not shown). Protein expression of total Rs6 did not change significantly (Figure 2.17). The phosphorylated form of Rs6 significantly increased in pigs that had been exposed to early gestation heat stress (Figure 2.18;  $p < 0.05$ ). No significant differences were observed in the ratio of p-Rs6:total Rs6 (data not shown). Pigs exposed to late gestation heat stress displayed a tendency ( $p < 0.10$ ) for increased total EEF2 protein expression (Figure 2.15), but there were no significant differences observed in the abundance of p-EEF2 (Figure 2.16). The ratio of p-EEF2:total EEF2 was not significantly different (data not shown).

## Discussion

It has been well established that heat stress conditions decrease the rate of metabolism in many of our livestock species including pigs. Pigs are some of the most susceptible animals to heat stress as a result of their limited heat dissipation capacities (Renaudeau et al., 2011). Our results are consistent in describing decreased metabolic efficiency and rate in pigs exposed to heat stress. This may be a result of a decrease in the activity of the mTOR pathway (Polak and Hall, 2009). The results of this study indicate that downstream targets of mTOR are changing in response to heat stress conditions.

The Akt enzyme is one of the first responders in the mTOR pathway. It is directly responsible for phosphorylation and activation of mTOR along with many other downstream protein complexes. Results from this study indicate that there are no significant differences in protein abundance of Akt or p-Akt in heat stressed pigs during the acute trial. However, there was a significant difference in total Akt abundance in pigs that have been chronically heat stressed. Specifically, there was an increase in total Akt abundance in pigs that had been exposed to heat stress prenatally during the second half of gestation. This is not paired with a subsequent increase in p-Akt, meaning that there is no increase in the active form of the enzyme.

Two of the downstream enzymes, 4eBP1 and Rs6, may be activated by the mTOR complex (Dann and Thomas, 2006). There were significant differences in the protein abundance of both during the acute and chronic heat stress studies. During the acute study, there was a significant decrease in p-4eBP1 in response to postnatal heat stress suggesting that there was less recruitment of mRNA to the ribosomal complex for

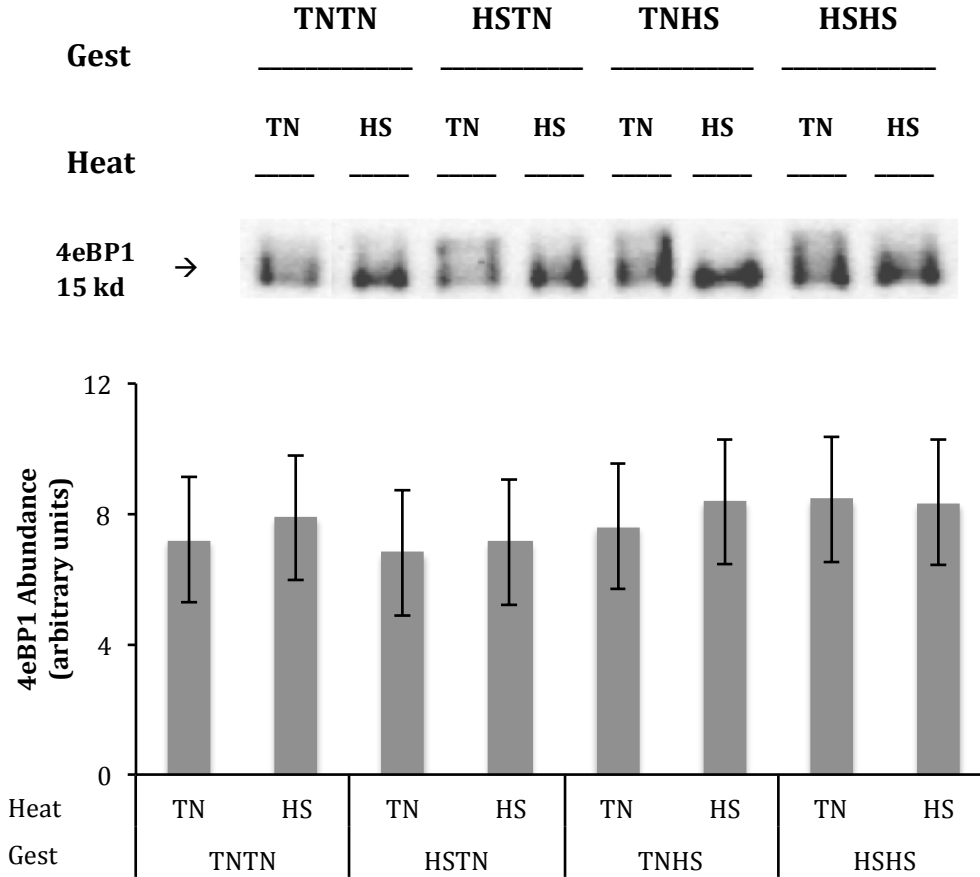
translation. Protein abundance of Rs6 was not only affected by postnatal heat stress, but also by gestational heat stress. Pigs that had been prenatally heat stressed for the second half of gestation and swine that had been prenatally heat stressed for the entirety of gestation showed significant decrease in total Rs6. It is directly involved in the initiation of protein synthesis. Some studies have shown that p-Rs6 does not change significantly with heat stress, which is consistent with these data (Richter et al., 1983). However, there is also a body of data suggesting that heat stress, separate from energy availability, may still affect phosphorylation of Rs6 (Tas and Martini, 1987). This particular study found that the rate of protein synthesis was decreased in heat shocked HeLa cells, but that the abundance of p-Rs6 may not have contributed to this decrease directly. It is unclear which theory is the correct one at this time, but the results shown here indicate that phosphorylation of Rs6 may not be affected by heat stress.

Decreases in total Rs6, as well as 4eBP1, provide a possible explanation for decreased protein deposition in pigs under heat stress. It appears that this effect is rapid, since the acute study lasted only 24 hours. This may in part be due to the lack of insulin signaling through the Akt pathway and the subsequent inactivation of mTOR. Insulin has been shown to induce S6 kinase phosphorylation in HeLa cells (Burger et al., 1988). With decreased feed consumption under heat stress conditions, there is less insulin secretion and less nutrient signaling through the mTOR pathway. This may be responsible for the decreases in protein synthesis observed in heat stressed animals.

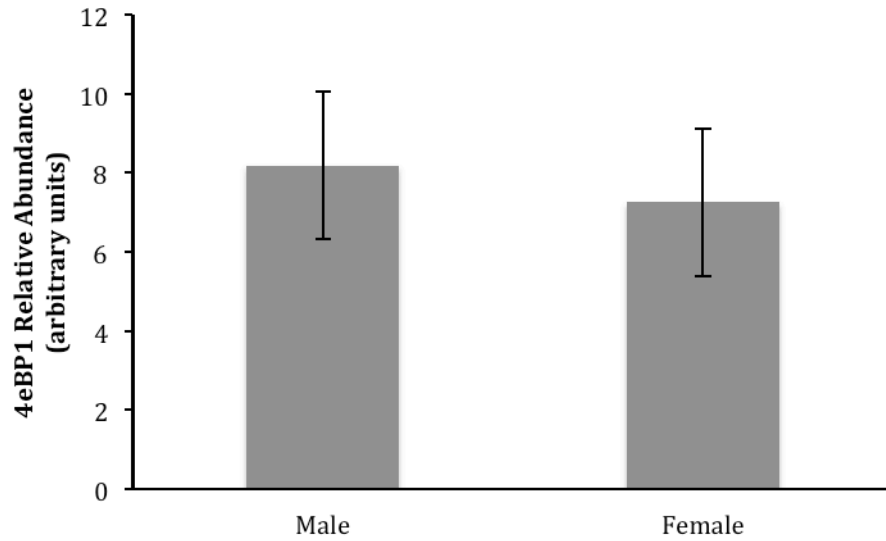
While there were still significant differences in 4eBP1 and Rs6 during the chronic study, the differences appear to be modified suggesting changes in metabolism with increased heat stress duration. The significant increase observed in total 4eBP1 during the

chronic trial seemed to be linked to prenatal heat stress in the second half of gestation. However, this was paired with a lack of significant differences in p-4eBP1. While there were no significant differences in total Rs6 observed during the chronic trial, there was a significant increase in p-Rs6 in pigs that had been prenatally heat stressed during the first half of gestation. It is possible that early gestational heat stress may have a priming effect on postnatal heat stress responses. This may allow animals to continue synthesizing vital proteins under harsh conditions (Duncan and Hershey, 1989; Kregel, 2002).

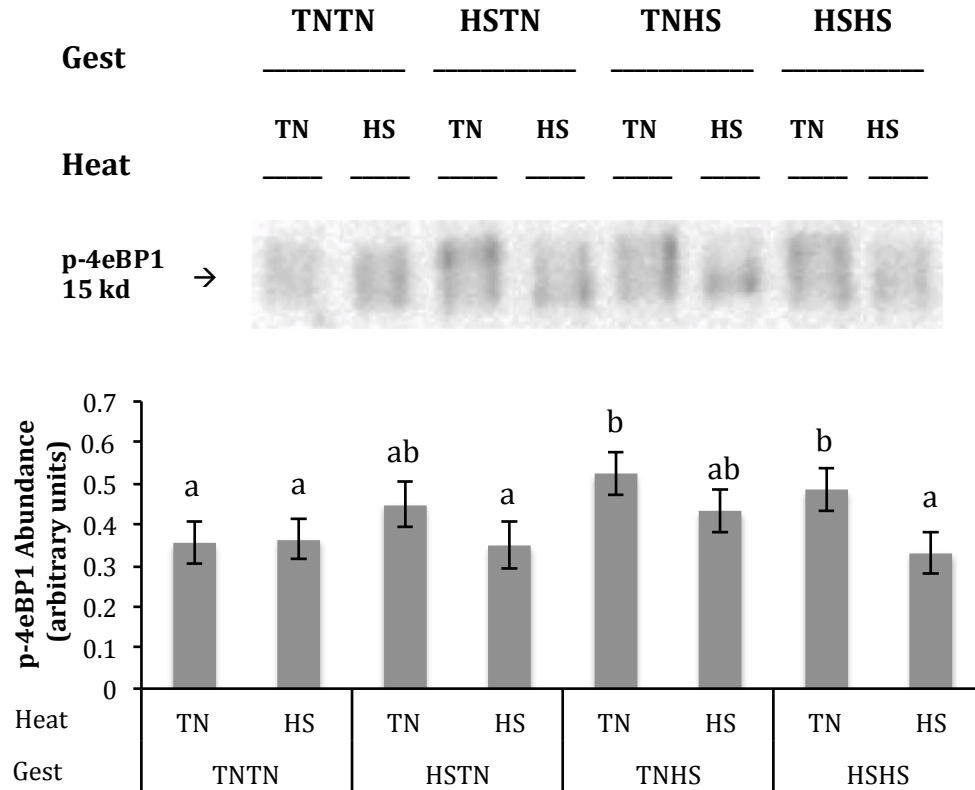
Surprisingly, there was no change in abundance of EEF2, though it has been thought to be another target of mTOR and is also involved with protein synthesis at the elongation phase. However, the aforementioned evidence does suggest that mTOR is not activating its major downstream targets as efficiently. This could affect not only protein synthesis for new enzymes at the molecular, but also protein synthesis for skeletal muscle development and growth. While studies have suggested that protein synthesis is sometimes increased in heat stressed animals, the results of this study indicate that the opposite may be true. Whether this effect is specific to pigs is something to be taken into consideration for further research.



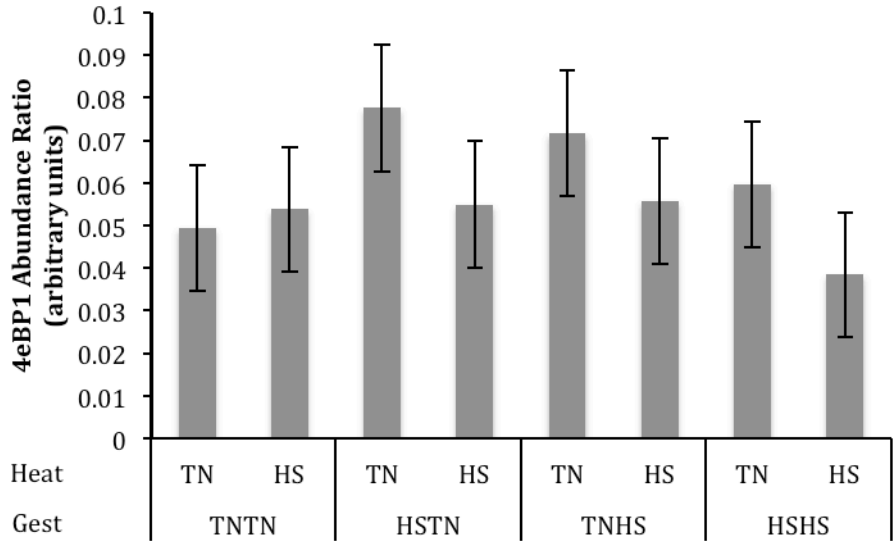
**Figure 2.1. Effects of acute heat stress on abundance of total 4eBP1 in skeletal muscle.** Top: Representative Western blot analysis of protein isolated from swine longissimus dorsi samples. Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or Heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 24 hours at 12 weeks of age. Bottom: Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



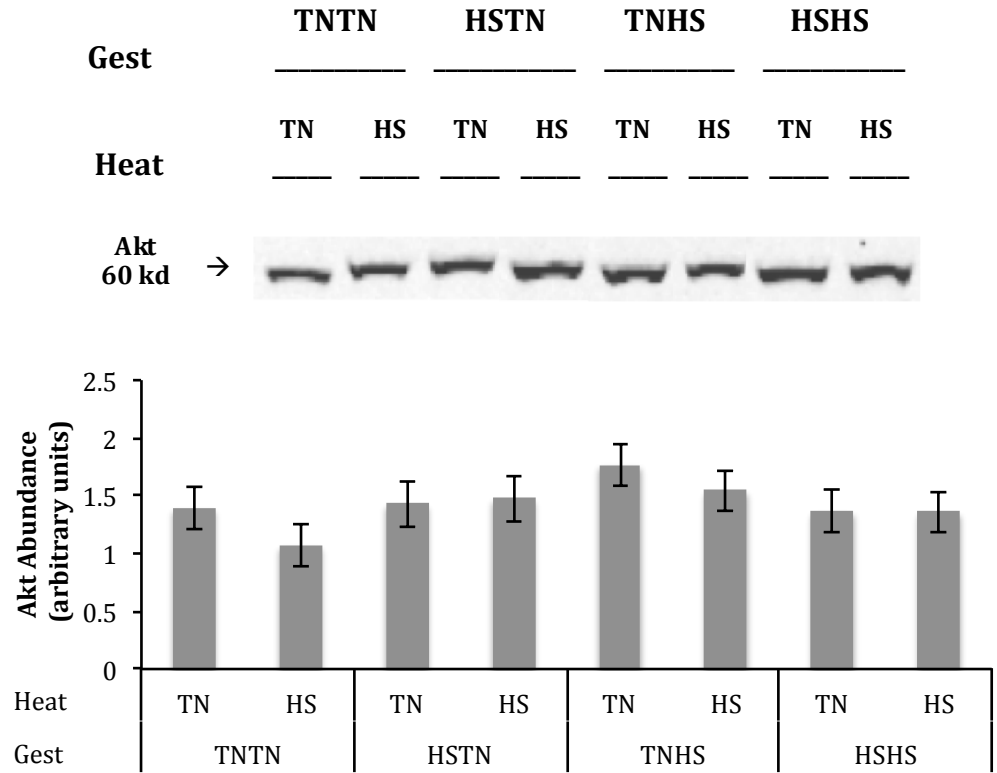
**Figure 2.2. Effects of sex on abundance of total 4eBP1 in swine under acute heat stress.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 24 hours at 12 weeks of age. Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis. Sex differences were found to be significant at  $p < 0.05$ .



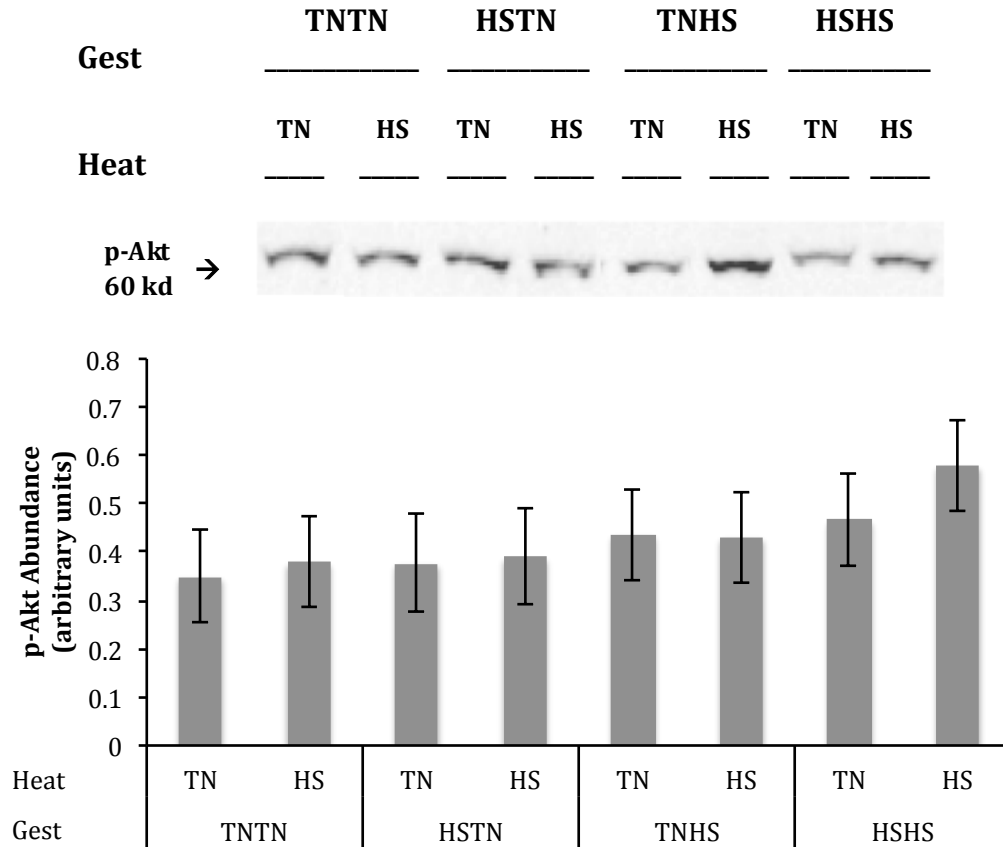
**Figure 2.3. Effects of acute heat stress on abundance of phosphorylated 4eBP1 in skeletal muscle.** Top: Representative Western blot analysis of protein isolated from swine longissimus dorsi samples. Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 24 hours at 12 weeks of age. Bottom: Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis. Different letters symbolize significant differences between treatments ( $p < 0.05$ ).



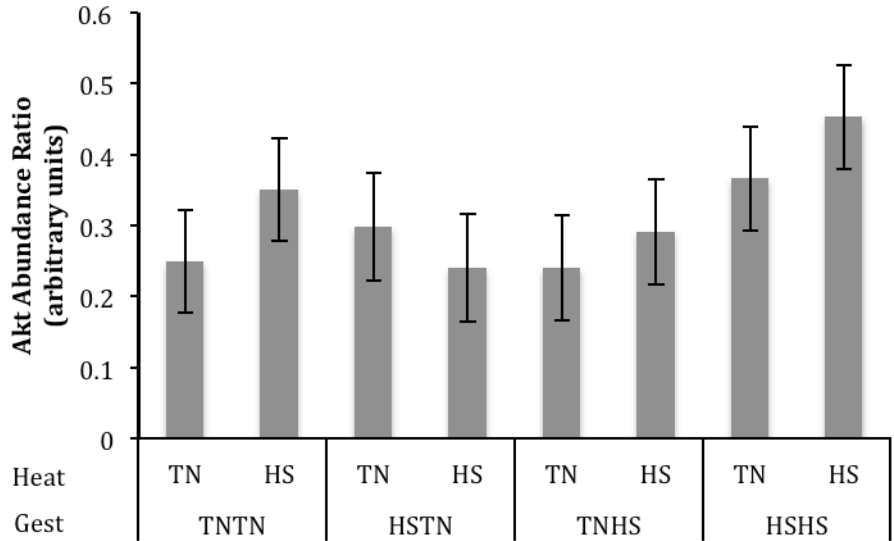
**Figure 2.4. Effects of acute heat stress on the ratio of the abundance of phosphorylated 4eBP1 to total 4eBP1 in skeletal muscle.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 24 hours at 12 weeks of age. Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis. A heat effect was observed with  $p < 0.05$ .



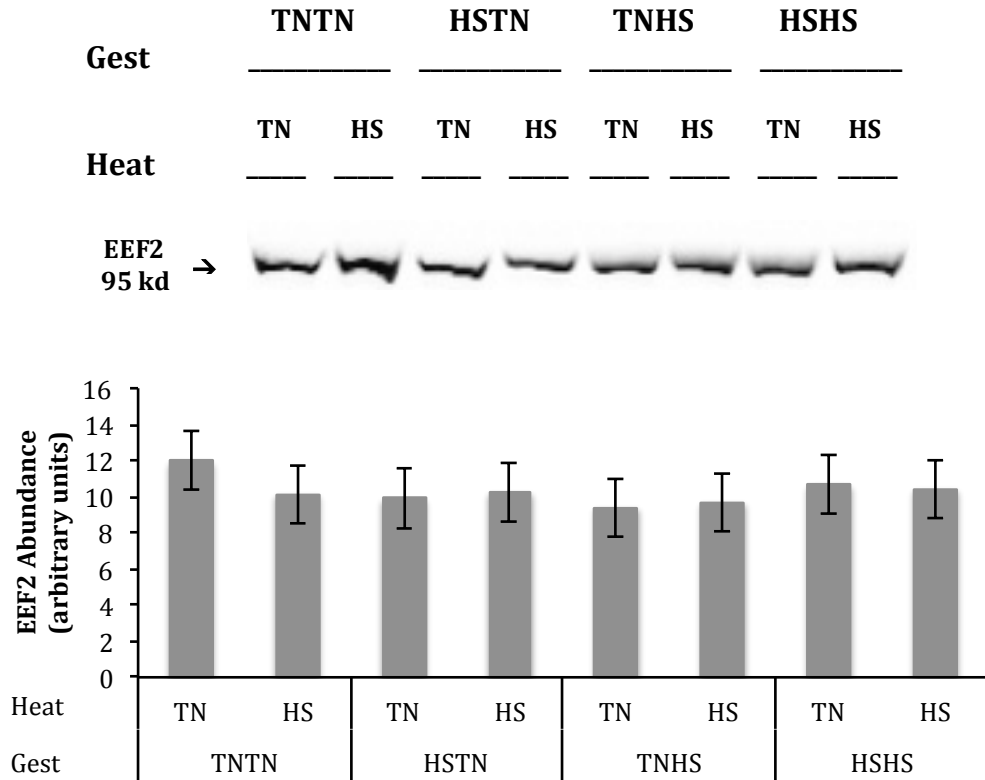
**Figure 2.5. Effects of acute heat stress on abundance of total Akt in skeletal muscle.** Top: Representative Western blot analysis of protein isolated from swine longissimus dorsi samples. Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 24 hours at 12 weeks of age. Bottom: Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



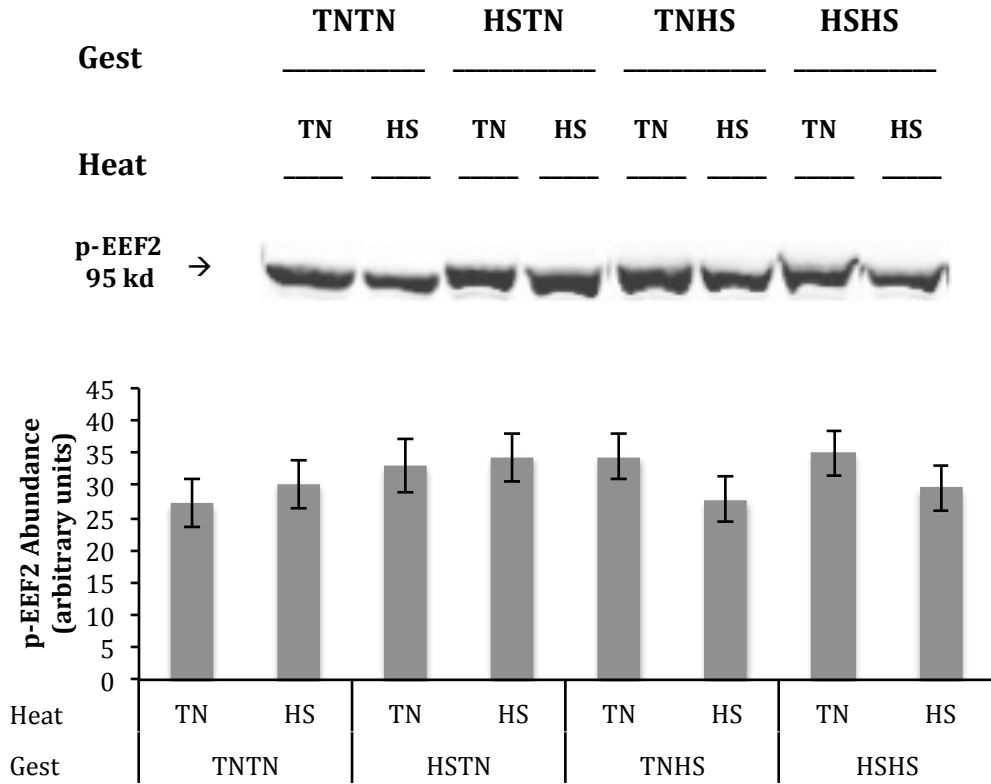
**Figure 2.6. Effects of acute heat stress on abundance of phosphorylated Akt in skeletal muscle.** Top: Representative Western blot analysis of protein isolated from swine longissimus dorsi samples. Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 24 hours at 12 weeks of age. Bottom: Raw abundance for each RNA sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



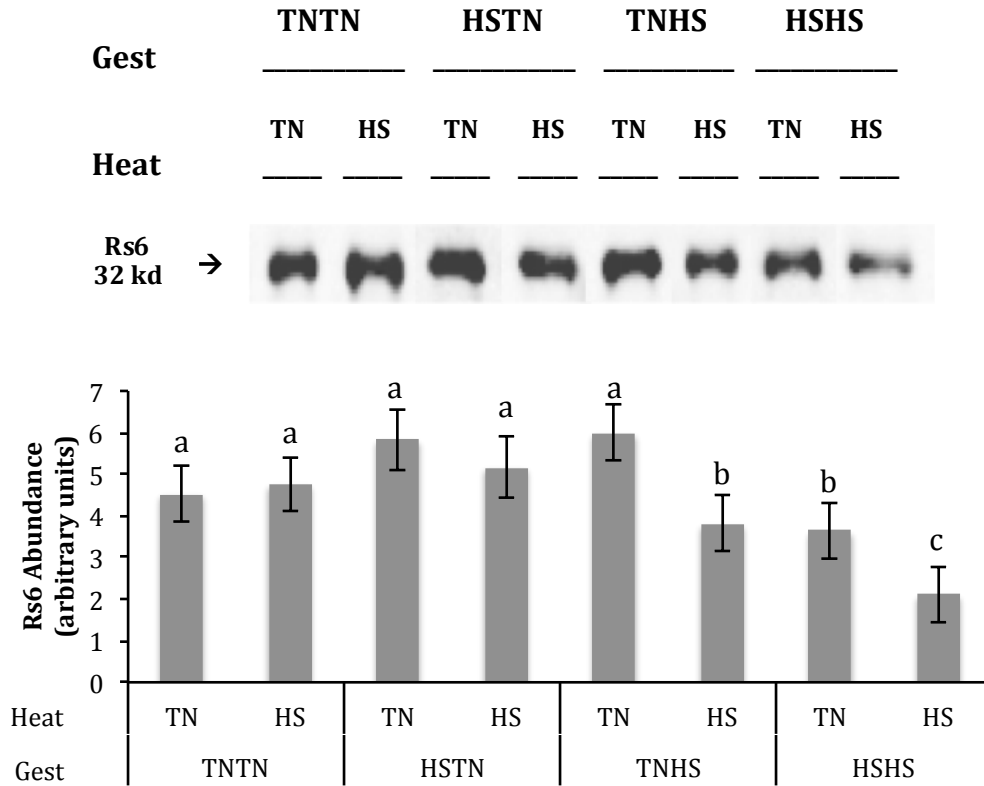
**Figure 2.7. Effects of acute heat stress on the ratio of the abundance of phosphorylated Akt to total Akt in skeletal muscle.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 24 hours at 12 weeks of age. Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis. A tendency for a heat effect was observed with  $p < 0.10$ .



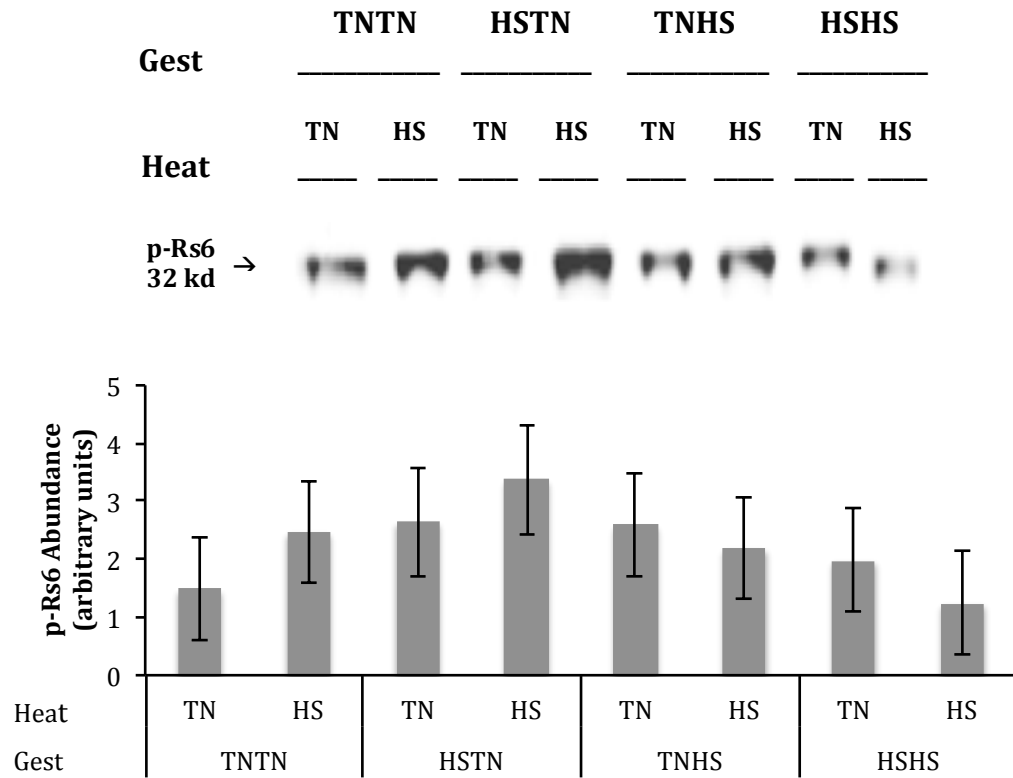
**Figure 2.8. Effects of acute heat stress on abundance of total EEF2 in skeletal muscle.** Top: Representative Western blot analysis of protein isolated from swine longissimus dorsi samples. Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 24 hours at 12 weeks of age. Bottom: Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



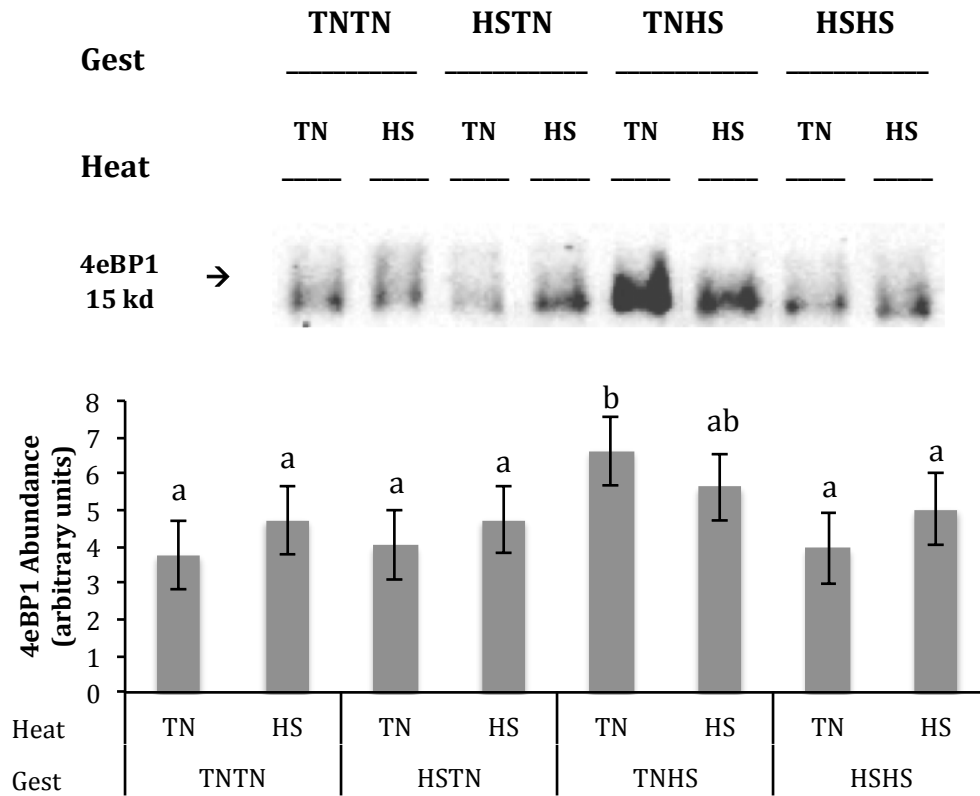
**Figure 2.9. Effects of acute heat stress on abundance of phosphorylated EEF2 in skeletal muscle.** Top: Representative Western blot analysis of protein isolated from swine longissimus dorsi samples. Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 24 hours at 12 weeks of age. Bottom: Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



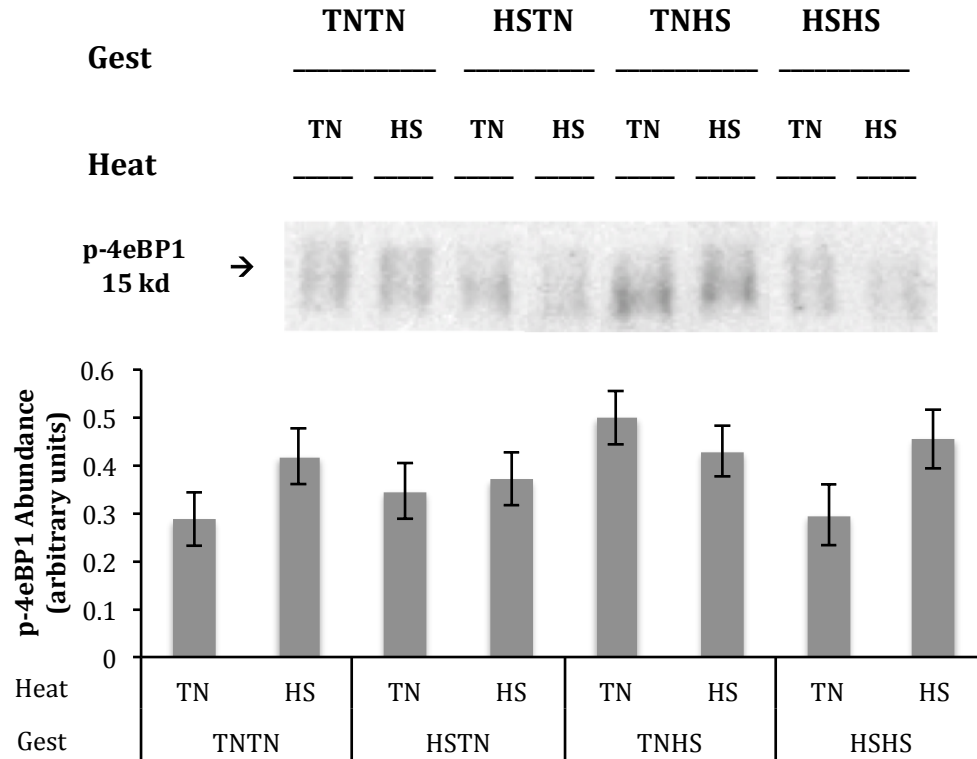
**Figure 2.10. Effects of acute heat stress on abundance of total Rs6 in skeletal muscle.** Top: Representative Western blot analysis of protein isolated from swine longissimus dorsi samples. Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 24 hours at 12 weeks of age. Bottom: Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis. Different letters symbolize significant differences between treatments ( $p < 0.05$ ).



**Figure 2.11. Effects of acute heat stress on abundance of phosphorylated Rs6 in skeletal muscle.** Top: Representative Western blot analysis of protein isolated from swine longissimus dorsi samples. Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 24 hours at 12 weeks of age. Bottom: Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.

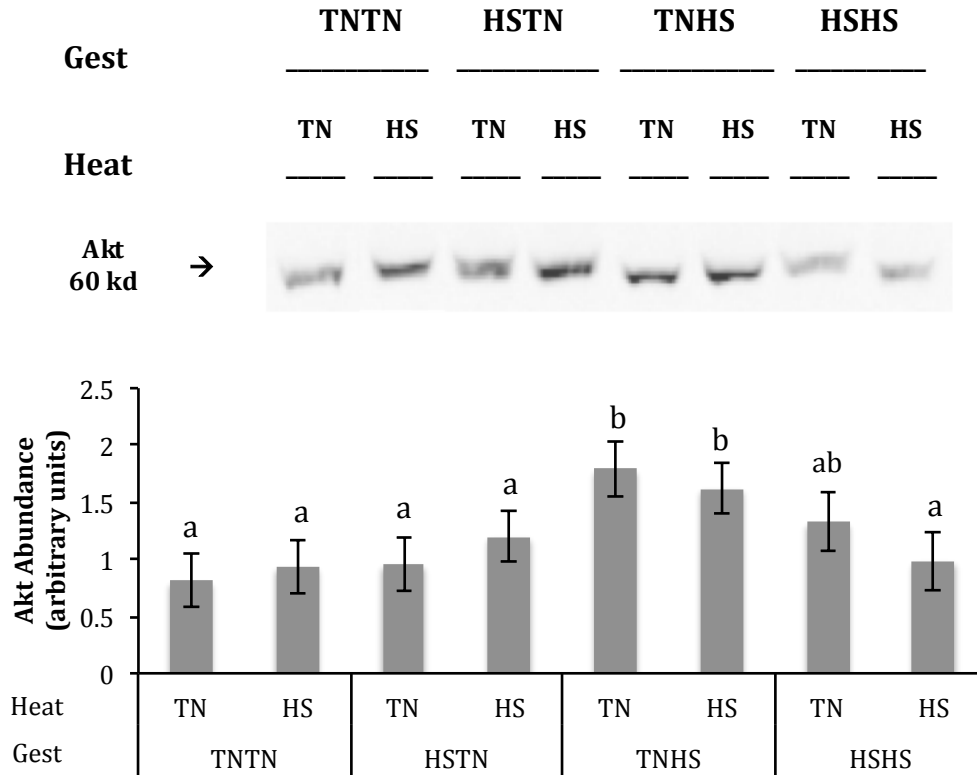


**Figure 2.12. Effects of chronic heat stress on abundance of total 4eBP1 in skeletal muscle.** Top: Representative Western blot analysis of protein isolated from swine longissimus dorsi samples. Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Bottom: Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis. Different letters symbolize significant differences between treatments ( $p < 0.05$ ).

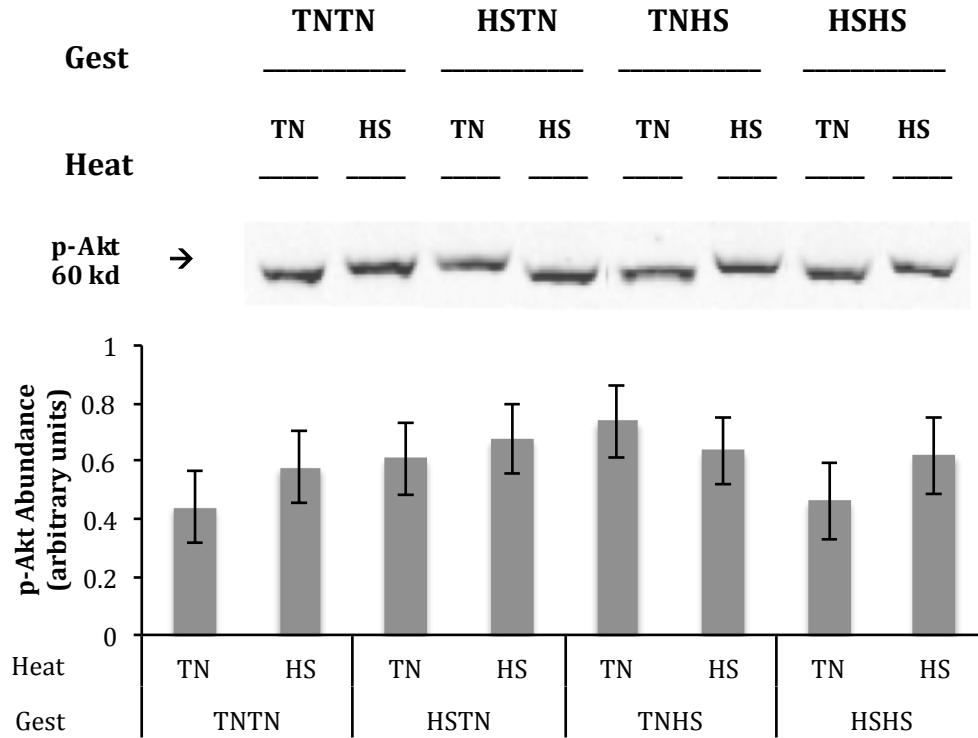


**Figure 2.13. Effects of chronic heat stress on abundance of phosphorylated 4eBP1 in skeletal muscle.**

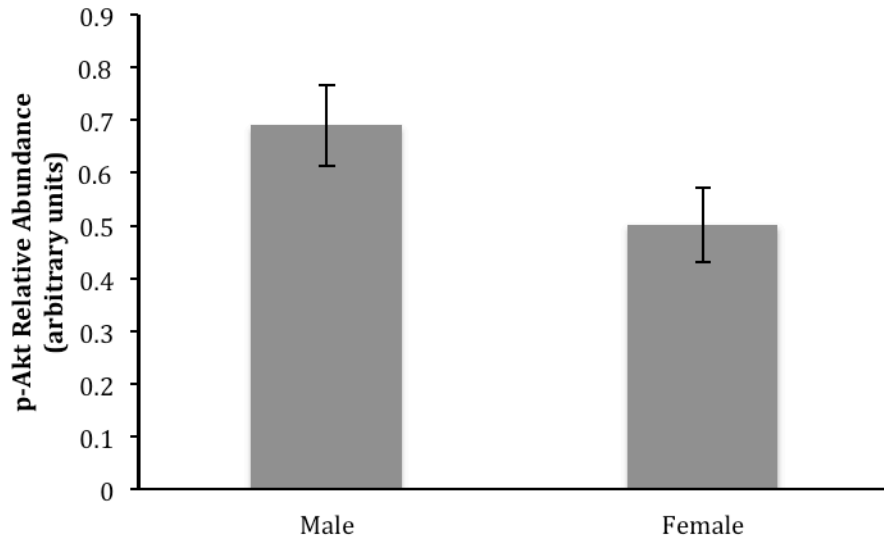
Top: Representative Western blot analysis of protein isolated from swine longissimus dorsi samples. Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Bottom: Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



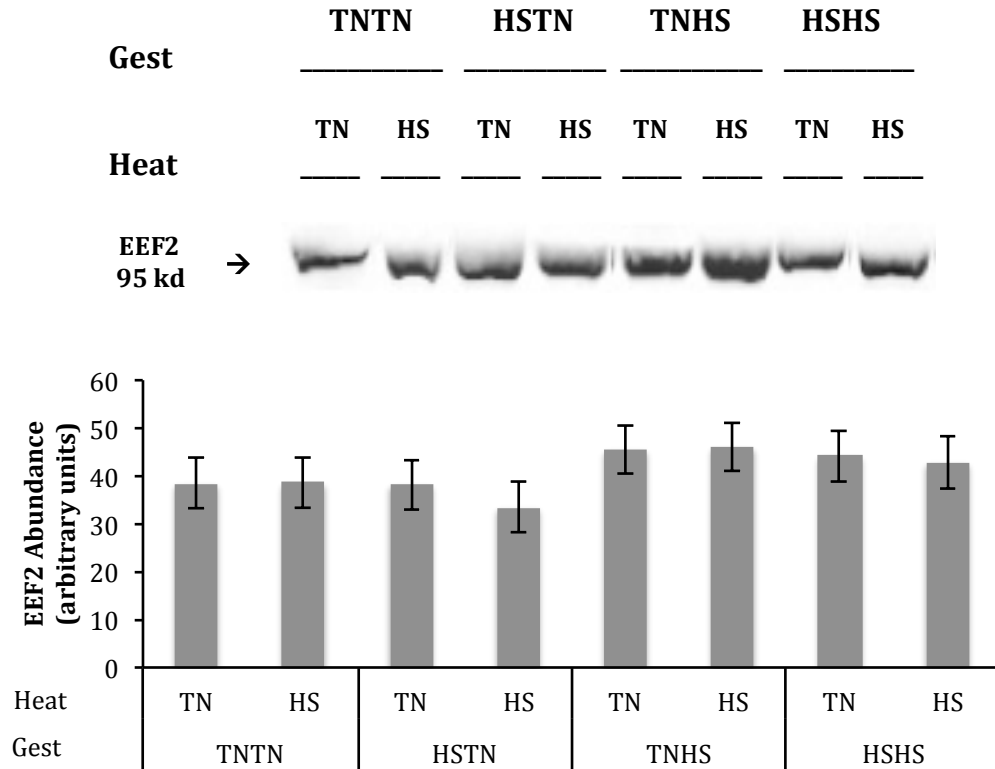
**Figure 2.14. Effects of chronic heat stress on abundance of total Akt in skeletal muscle.** Top: Representative Western blot analysis of protein isolated from swine longissimus dorsi samples. Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Bottom: Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis. Different letters symbolize significant differences between treatments ( $p < 0.05$ ).



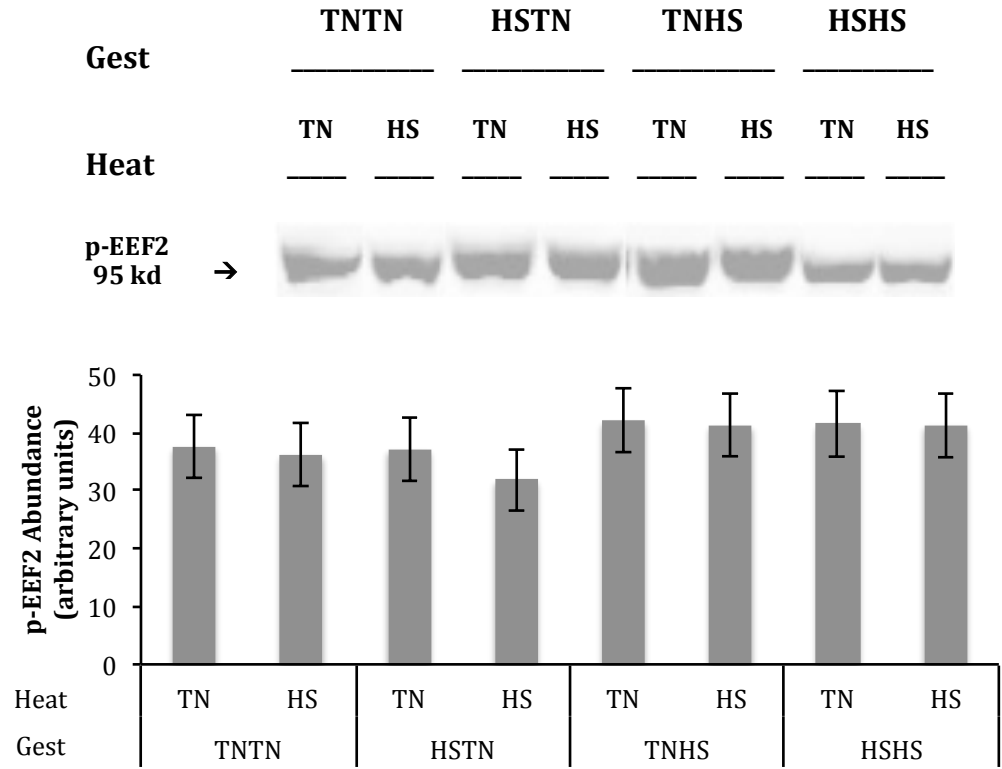
**Figure 2.15. Effects of chronic heat stress on abundance of phosphorylated Akt in skeletal muscle.** Top: Representative Western blot analysis of protein isolated from swine longissimus dorsi samples. Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Bottom: Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



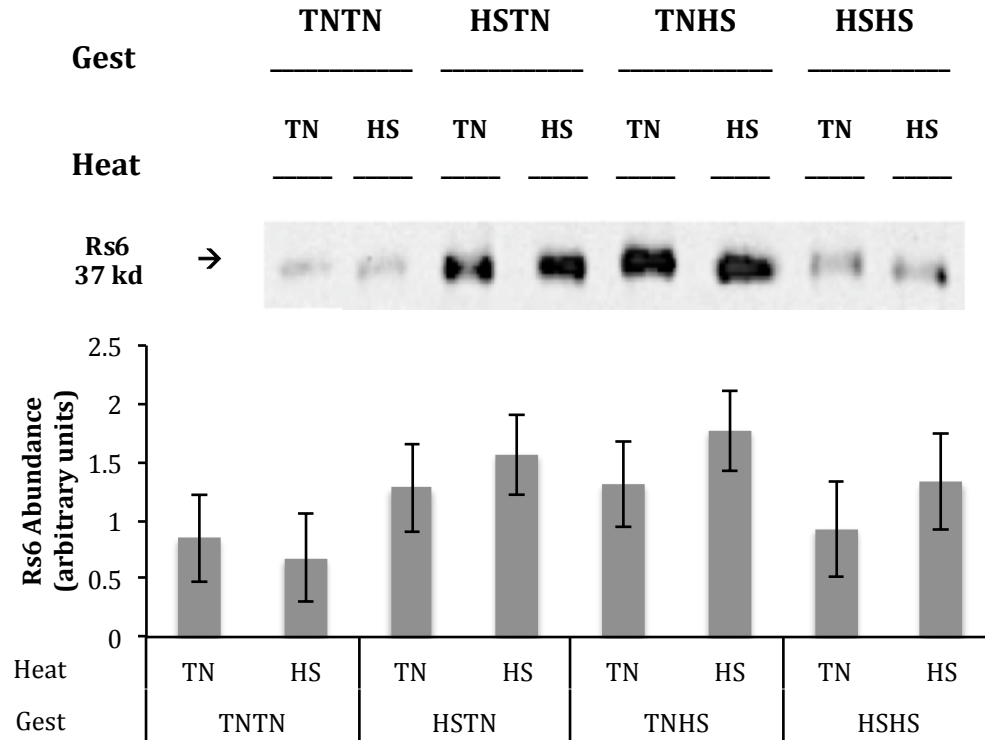
**Figure 2.16. Effects of sex on abundance of phosphorylated Akt in swine under chronic heat stress.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 24 hours at 12 weeks of age. Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis. Sex differences were found to be significant at  $p < 0.05$ .



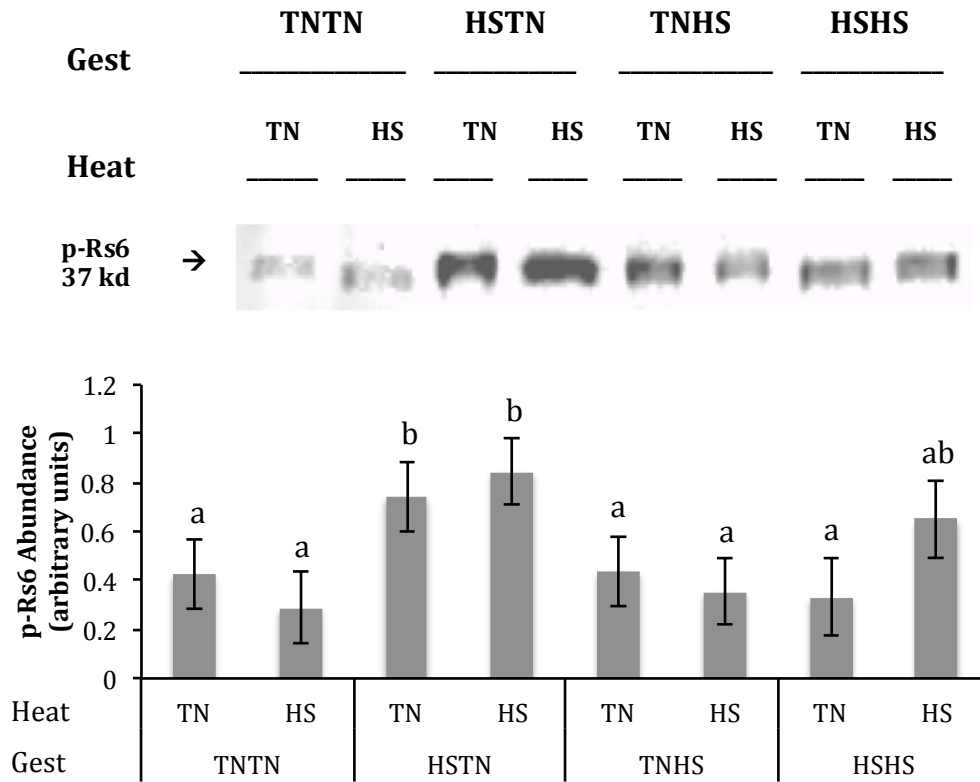
**Figure 2.17. Effects of chronic heat stress on abundance of total EEf2 in skeletal muscle.** Top: Representative Western blot analysis of protein isolated from swine longissimus dorsi samples. Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Bottom: Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



**Figure 2.18. Effects of chronic heat stress on abundance of phosphorylated EEF2 in skeletal muscle.** Top: Representative Western blot analysis of protein isolated from swine longissimus dorsi samples. Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Bottom: Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



**Figure 2.19. Effects of chronic heat stress on abundance of total Rs6 in skeletal muscle.** Top: Representative Western blot analysis of protein isolated from swine longissimus dorsi samples. Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Bottom: Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



**Figure 2.20. Effects of chronic heat stress on abundance of phosphorylated Rs6 in skeletal muscle.** Top: Representative Western blot analysis of protein isolated from swine longissimus dorsi samples. Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Bottom: Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis. Different letters symbolize significant differences between treatments ( $p < 0.05$ ).

## CHAPTER III

### Effects of acute and chronic heat stress on metabolic gene expression in swine

#### ABSTRACT

Heat stress is known to have detrimental effects on nutrient partitioning and metabolism across livestock species. Pigs may be particularly susceptible due to their inability to cope efficiently with heat dissipation. In order to assess the effects of heat stress on metabolism, sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were divided into two constant TN (21°C) or HS (35°C) treatment groups (n=48 per treatment) at 14 weeks of age for the acute heat stress study (duration of 24 hours) and the chronic heat stress study (duration of 5 weeks). Skeletal muscle samples (longissimus dorsi) were collected at the end of each treatment period. Significant decreases ( $p<0.05$ ) were observed in genetic expression of CytB, MURF, ND1, and TFAM based on postnatal heat treatment in acute heat stress samples. Gene expression of Cox5B, EEF2, HK2, PGC-1 $\alpha$ , SDHA, and TFAM decreased based on gestation treatment during the acute heat stress trial. A significant increase ( $p<0.05$ ) was observed in HK2 based on postnatal heat treatment in both acute and chronic samples. Significant decreases ( $p<0.05$ ) were observed in genetic expression of CytB, MURF, and PGC-1 $\alpha$ , based on postnatal heat treatment during the chronic heat stress trial. These data suggest that there may be both immediate and prolonged effects of heat stress on metabolic gene expression in swine.

## Introduction

Animals under heat stress conditions are known to display a variety of differences in energy metabolism. Heat stress decreases voluntary feed intake and activity level, allowing the animal to slow the rate of heat production. However, decreases in feed consumption can result in negative impacts on growth and muscle deposition (Boddicker et al., 2012). Swine are of particular interest in heat stress studies due to their inability to effectively dissipate heat via sweating. This causes them to be more sensitive to heat stress conditions, which must be taken into account by the producer.

Metabolism may be affected by heat stress in a variety of ways. One of the most interesting may be the shift of pathway from energetically efficient oxidative phosphorylation to the less efficient glycolysis. There is also a shift away from protein deposition and towards fat deposition. Boddicker et al. (2012), report that heat stressed swine display increased back fat depth with prenatal exposure to heat stress conditions during the first half of gestation. At the same time, feed intake and weight gain were observed to decrease significantly with postnatal heat stress providing further evidence for a concurrent decrease in metabolic rate.

There are many enzymes related to metabolism that may be affected by heat stress conditions. This study examines gene and protein expression for only a few of these enzymes, and uses them as markers to determine the effect of heat stress on skeletal muscle metabolism in swine for both short and long term periods. It seeks to address the research question: How does the shift in body composition from protein to lipid occur? While some of these enzymes are related to nutrient intake and signaling, others are

involved with specific metabolic pathways allowing for the determination of active pathways during acute and chronic heat stress in swine.

## **Materials and Methods**

### ***Animals and experimental design***

Animal gestational and heat stress experiments were performed at the University of Missouri and Iowa State University in a manner identical to that described in Chapter II.

### ***Protein extraction and Western blots***

Protein extraction, BCA assays, and Western blots were performed in a manner identical to that described in Chapter II. Primary antibodies used for Western blots in this study included GAPDH and PKM2 from Cell Signaling, and PDH and p-PDH from Calbiochem.

### ***RNA extraction***

Longissimus dorsi (LD) muscle samples, collected in Iowa and shipped to Virginia Tech were transferred from a freezer set at -80°C to liquid nitrogen. Samples were kept frozen as they were crushed in the original foil package, transferred to new Whirl-Pack bags, and placed back into liquid nitrogen for extraction. Approximately 100 mg of sample were transferred to a 5 mL plastic centrifuge tube containing 1 mL of Trizol cell lysis reagent (5 Prime, Gaithersburg, MD). Tissue was homogenized in the

Trizol reagent using a Power Gen 125 homogenizer from Fisher Scientific set at six speed for two passes of approximately 15 seconds each. The homogenizer was cleaned with deionized water, and primed with Trizol between each sample. 200  $\mu$ L of chloroform were added to the cell lysate and vortexed for approximately 15 seconds creating an emulsion. The emulsion was transferred to a phase lock gel tube from 5 Prime (Gaithersburg, MD) and incubated at room temperature for 5 minutes. The phase lock gel tube was centrifuged for 5 minutes at 15000 x g, separating the clear aqueous phase and the pink colored organic phase. The clear aqueous phase, left on the top of the gel, was transferred to a new 1.5 mL centrifuge tube. 500  $\mu$ L of isopropanol were added to the aqueous phase. Tubes were inverted to mix, and incubated on ice for 5 minutes in order to allow the RNA to precipitate. Tubes were then transferred to a centrifuge set at 4°C and spun for 10 minutes at 15,000 x g. The supernatant was poured off leaving the RNA pellet at the bottom of the tube. 1 mL of 70% ethanol was then added to the RNA pellet. Tubes were spun again at 15,000 x g for 5 minutes. The supernatant was poured off. Excess supernatant was gently pipetted from around the RNA pellet in order to allow it to dry thoroughly. The RNA pellet was incubated on ice, and exposed to air for 5 minutes. 100  $\mu$ L of RNase free water were added to the pellet and mixed in order to redissolve the total RNA. Once the RNA pellet was seen to be partially dissolved, the tubes were transferred to the -80°C freezer to be stored until RNA Clean-Up.

### ***RNA clean-up***

Total RNA samples were retrieved and thawed from the -80°C freezer. Samples were mixed thoroughly in order to ensure that all RNA was dissolved into the RNase

free water. A Nanophotometer Pearl (Implen, Westlake Village, CA) was used to determine purity and concentration of total RNA in each sample. Samples exceeding 900 ng/ $\mu$ L in concentration were diluted with RNase free water to approximately 800 ng/ $\mu$ L for clean up. All samples used in the clean up procedure were 100  $\mu$ L by volume. The Qiagen RNEasy CleanUp Kit was used for this procedure. 350  $\mu$ L of  $\beta$ -mercaptoethanol diluted in RLT Buffer (10  $\mu$ L  $\beta$ -mercaptoethanol to 350  $\mu$ L RLT Buffer) was added to each sample. 250  $\mu$ L of 100% ethanol was added to each sample and mixed by pipetting. Each sample was then transferred to an RNEasy spin column and spun for 15 seconds at 10,000 x g. The flow through collected at the bottom of the column was discarded. 350  $\mu$ L of RW1 Buffer was added to each column, and columns were spun again for 15 seconds at 10,000 x g. 80  $\mu$ L of DNase, diluted in RDD Buffer (10  $\mu$ L DNase to 70  $\mu$ L RDD buffer) was added to each column and incubated for 15 minutes at room temperature. 350  $\mu$ L of RW1 Buffer were then added to each column, and columns were spun for 15 seconds at 10,000 x g. Flow through was discarded. 500  $\mu$ L of RPE Buffer were added to each spin column. Columns were spun for 15 seconds at 10,000 x g. 500  $\mu$ L of RPE Buffer were added to each spin column. Columns were spun for 2 minutes at 10,000 x g. After this wash procedure, the flow through tube was discarded, and the spin column was transferred to a new flow through tube. The column was spun once more at 15,000 x g for 1 minute to get rid of any excess ethanol that was left behind. Columns were then transferred to a 1.5 mL centrifuge tube. 50  $\mu$ L of RNase free water were added to the column, and columns were spun for 1 minute at 10,000 x g. The flow through was carefully pipetted from the centrifuge tube back into the column. Columns were spun once more for 1 minute at 10,000 x g. Samples in the centrifuge tubes were examined

using the nanophotometer for concentration and purity, and then stored in the -80°C freezer.

### ***cDNA synthesis***

The Bio-Rad iScript Advanced cDNA Synthesis Kit for RT-qPCR was used for this procedure. Purified total RNA samples were thawed from the -80°C freezer. 5 µg/uL RNA were used for each cDNA synthesis reaction. Samples were diluted with RNase free water to a total volume of 5 µL. 15 µL of a master mix containing the iScript advanced reaction mix and the iScript advanced reverse transcriptase were added to each diluted sample. Reverse pipetting was used to mix each sample. The total volume for each reaction was 20 µL. Sample tubes were incubated using a Bio-Rad thermal cycler set for 30 minutes at 42°C followed by 5 minutes at 85°C and a holding period set for 4°C. cDNA samples were then frozen and stored at -20°C until RT-qPCR.

### ***Real time-qPCR***

Gene expression analysis was performed using real-time PCR as described previously (Rhoads et al., 2011). The SsoAdvanced SYBR Green Supermix Kit from Bio-Rad was used for this procedure. 12.5 µl SYBR Green, 10 µl (1ng/µl) of cDNA, 0.625 µl (10µM) of forward primer, and 0.625 µl of (10µM) of reverse primer (primer information included in Table 1) were mixed RNase free water to a total volume of 25 µL per reaction. Samples were incubated using a Bio-Rad CFX96 Real Time System set for enzyme activation at 95°C for 30 seconds (1 cycle), denaturation at 95°C for 5 seconds (40 cycles), annealing and extension at 65°C for 30 seconds (40 cycles), and a

melt curve from 65-95°C in 5°C increments. Analyses of amplification plots were performed with CFX Manager 2.1 Software (BioRad). The fold differences between the internal control sample and the samples of interest were calculated with the amplification efficiencies of each respective target gene (amplification efficiency taken to the power of the control  $C_T$  minus the sample  $C_T$ ).

### ***Data analysis***

All protein data were analyzed using the proc mixed procedure of SAS as described in Chapter II.

Gene expression data were analyzed using the proc mixed procedure of SAS. Analysis for gene expression was based off of the equation:  $Y_{ijklm} = \mu + \alpha_i + \beta_j + \gamma_k + (\beta\gamma)_{jk} + \delta_{l(j)} + \varepsilon_{ijklm}$ , where  $\mu$  represents the overall mean of the population,  $\alpha_i$  represents the mean effect of sex,  $\beta_j$  represents the mean effect of gestation treatment,  $\gamma_k$  represents the mean effect of heat treatment,  $(\beta\gamma)_{jk}$  represents the interaction of gestation and heat treatments,  $\delta_{l(j)}$  represents the random effect of sow, and  $\varepsilon_{ijklm}$  represents the unexplained residual element assumed to be independent and normally distributed. Results were considered to be significant with a p-value less than 0.05.

## **Results**

### ***Acute heat stress results***

Gene expression of enzymes associated with energy metabolism appeared to be rapidly affected by heat stress. HK2 gene expression increased significantly in pigs exposed to acute heat stress (Figure 3.13;  $p < 0.05$ ). There was also a significant decrease

in HK2 in pigs that had been exposed to heat stress throughout gestation (Figure 3.13;  $p < 0.01$ ). There was a tendency ( $p < 0.10$ ) observed for PDK4 expression to increase in swine that had been exposed to heat stress compared to pigs that had been maintained in thermoneutral conditions (Figure 3.19). Gene expression of SDHA was also significantly decreased in pigs that had been exposed to heat stress throughout gestation (Figure 3.21;  $p < 0.01$ ). There was also a tendency ( $p < 0.10$ ) for gene expression of SDHA to decrease in pigs that had been heat stressed postnatally (Figure 3.21).

Genes associated with the process of oxidative phosphorylation and the electron transport chain were drastically affected by acute heat stress. A highly significant decrease was seen in ND1 expression in pigs exposed to heat stress during the 24 hour acute trial (Figure 3.18;  $p < 0.001$ ). Gene expression of CytB also showed significant decrease (Figure 3.11;  $p < 0.05$ ) along with gene expression of Cox5B (Figure 3.10;  $p < 0.01$ ).

MURF gene expression was significantly decreased in animals that had been heat stressed compared to those that had not (Figure 3.16;  $p < 0.05$ ). There was also a tendency for MURF to increase in pigs that had been exposed to early gestation heat stress (Figure 3.16). PGC-1 $\alpha$  gene expression significantly decreased in animals that had been exposed to heat stress throughout gestation (Figure 3.20;  $p < 0.05$ ). Finally, gene expression of TFAM was observed to significantly decrease in pigs exposed to heat stress postnatally ( $p < 0.01$ ) as well as in pigs that were exposed to heat stress at any point during gestation (Figure 3.22;  $p < 0.001$ ).

No significant differences were observed in HSP70 (Figure 3.15) or ATR gene expression (Figure 3.9) during the acute trial. No significant differences were observed in

levels of GAPDH, PDH, p-PDH or PKM2 protein abundance during the acute heat stress trial (Figures 3.1, 3.2, 3.3, 3.4).

### ***Chronic heat stress results***

Many of the differences seen in gene expression in the acute trial were not observed in the chronic trial. ATR gene expression did not change with chronic heat stress (Figure 3.23). There was a tendency ( $p < 0.10$ ) for HSP70 gene expression to increase in pigs exposed to heat stress throughout gestation when exposed to postnatal heat stress (Figure 3.27). Pigs not exposed to heat stress during gestation were observed to have a tendency ( $p < 0.10$ ) towards decreased HSP70 gene expression when exposed to postnatal heat stress (Figure 3.27).

No significant differences were observed in HK2 (Figure 3.26), PDK4 (Figure 3.30), or SDHA (Figure 3.32). However, there was a tendency ( $p < 0.10$ ) for ND1 to decrease in pigs exposed to gestational heat stress (Figure 3.29). CytB gene expression was significantly decreased ( $p < 0.05$ ) in response to chronic heat stress (Figure 3.25). Contrary to data from the acute heat stress study, however, Cox5B gene expression did not appear to change with chronic heat stress (Figure 3.24).

A few of the genes known to affect mitochondrial biosynthesis and other related processes were shown to be affected by chronic heat stress. MURF gene expression was seen to decrease significantly (Figure 3.28;  $p < 0.01$ ). PGC-1 $\alpha$  was observed to significantly increase with chronic heat stress (Figure 3.31;  $p < 0.05$ ). There was also a tendency ( $p < 0.10$ ) observed for PGC-1 $\alpha$  gene expression to increase with exposure to

gestational heat stress (Figure 3.31). TFAM expression did not appear to change with chronic heat stress (Figure 3.33).

No significant changes were observed for protein abundance of PDH, p-PDH or PKM2 during the chronic heat stress trial (Figures 3.6, 3.7, 3.8). There was a tendency ( $p < 0.10$ ) for GAPDH expression to increase with late gestation heat stress, but no other significant differences were observed (Figure 3.5).

## Discussion

Interestingly, there were no significant differences observed in protein abundance of PDH and PKM2 in either the acute or the chronic trials. The PDH enzyme converts pyruvate to acetyl-coA for entry into the TCA cycle, and PKM2 creates pyruvate as the final step of glycolysis. It is surprising that these proteins appear to be unaffected by heat stress. GAPDH was unaffected in the acute heat stress study, but tended to increase during the chronic heat stress study in swine that had been exposed to late gestation heat stress. The GAPDH enzyme catalyzes the sixth step of glycolysis, converting glyceraldehyde-3-phosphate into glycerate 1,3-bisphosphate. These protein abundance results are interesting since it would generally be expected that proteins such as PDH and PKM2 would increase, shifting energy metabolism towards glycolytic pathways. The tendency for GAPDH to increase could potentially signify an increase in glycolytic rate, but more research is necessary in order to confirm this.

Despite a lack of differences observed in protein expression, gene expression for a variety of enzymes seemed to be affected by heat stress both in the acute and chronic

studies. Significant increases in HK2 were observed in swine during the acute study. The HK2 enzyme commits glucose to the glycolytic pathway. Increases in this enzyme signify a concurrent increase in the overall rate of glycolysis, and the importance of glucose as a fuel source in pigs over 24 hours. It is important to note that this shift was not observed in the chronically heat stressed animals. It is possible that there may be an adaptive process occurring which may allow swine to maintain a consistent metabolic rate under chronic heat stress conditions. A different pattern was observed in SDHA gene expression, with significant decreases observed in the acute study only. The SDHA enzyme is responsible for converting succinate to fumarate as part of the TCA cycle. This process provides electrons for entry into oxidative phosphorylation. Decreases in SDHA imply that there is a decrease in oxidative phosphorylation, consistent with the idea that metabolic rate slows in heat stressed animals. Adaptation to heat stress over the five week chronic study may again be responsible for the lack of differences observed in those animals.

The PDK4 enzyme has the function of decreasing overall metabolism and shutting down the PDC. There was a tendency for PDK4 to increase during the acute heat stress study indicating that there may have been some decrease in the rate of metabolism through the TCA cycle and an increase in glycolysis. This is consistent with results obtained for other genes within the acute study. Again, it must be noted that there was no significant difference observed for PDK4 during the chronic study suggesting that metabolic rate in these animals was close to normal.

Heat stressed animals displayed more sensitivity to heat stress through gene expression for enzymes directly related to the electron transport chain and oxidative

phosphorylation. ND1, Cox5B, and CytB were all significantly decreased in heat stressed animals during the acute study. Chronically heat stressed animals displayed a tendency for decreased ND1, and significant decreases in CytB. However, there was no change seen in Cox5B, the terminal enzyme in the electron transport chain. Despite this, it appears that heat stress does decrease metabolism through oxidative phosphorylation, potentially in favor of glycolysis and other alternative pathways.

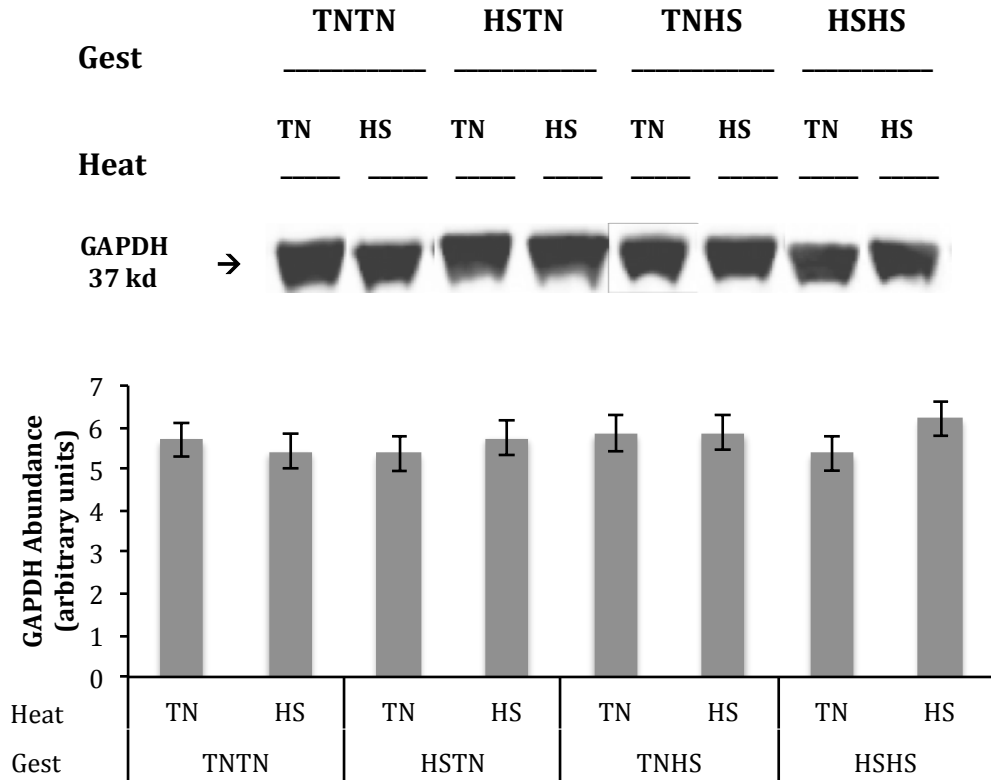
The gene expression of PGC-1 $\alpha$  was significantly decreased in acute heat stress animals and significantly increased in chronic heat stress animals. Since PGC-1 $\alpha$  is involved with muscle fiber type determination and also with mitochondrial biogenesis (Wenz et al., 2008; Hood, 2009), it is possible PGC-1 $\alpha$  may follow similar patterns to the other genes described previously. Heat stress over 24 hours may create a rapid, immediate degradation of muscle tissue along with the decreasing rates of energy metabolism required to support muscle fiber synthesis and protein deposition. Over time, however, animals may become acclimated to constant heat stress conditions and resume consistent metabolism (Duncan and Hershey, 1989; Moseley, 1997). The increase observed in PGC-1 $\alpha$  may even be consistent with studies suggesting that a certain degree of heat stress can increase muscle build up (Kamanga-Sollo et al., 2011). Further investigation is required to confirm these implications. The gene expression of TFAM, another gene related to mitochondrial biosynthesis was observed to decrease significantly in the acute, but not the chronic heat stress trial. This remains consistent with the rest of this study in that adaptation to heat stress may allow for the resumption of stable metabolic rate and protein synthesis.

Gene expression of MURF was significantly decreased in both acute and chronic heat stress samples. It has been proposed that MURF regulates metabolism under stress conditions (Koyama et al., 2008). This is especially true of amino acid metabolism and protein turnover. Decreases in MURF may indicate shifts of metabolic pathway in regards to skeletal muscle synthesis and degradation.

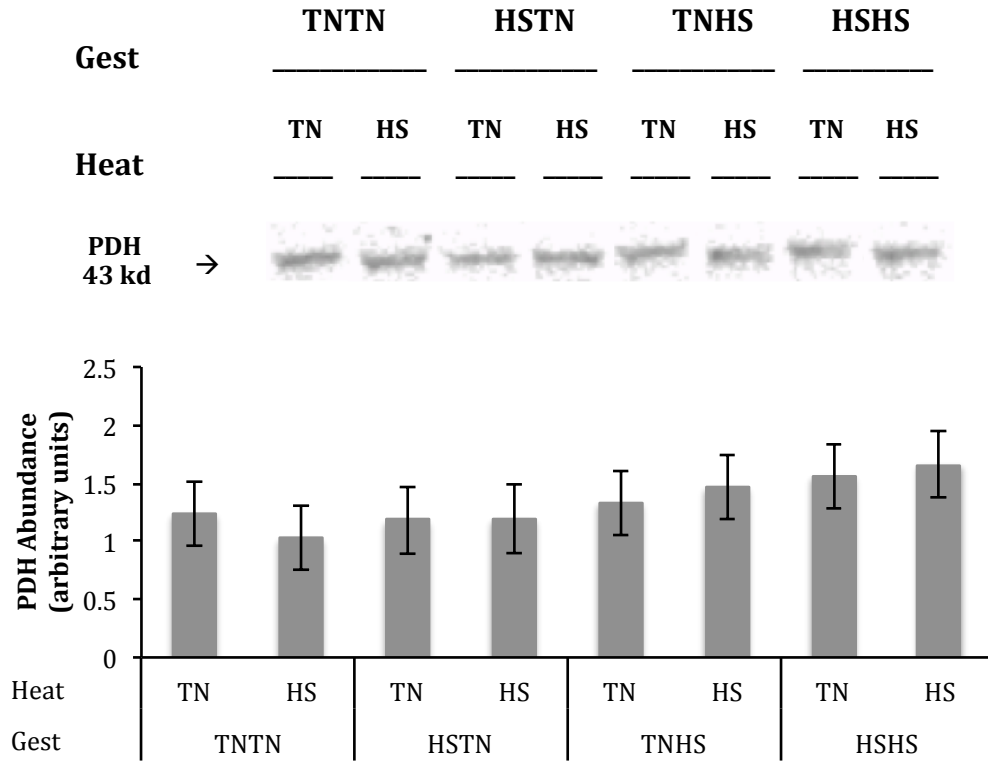
Surprisingly, HSP70 was not observed to increase in the acute study. There was only a tendency for an increase observed in the chronic study as well. Heat shock proteins are known to increase under heat stress conditions as a protective mechanism. It is possible that HSP70 may require more time to mobilize a response than 24 hours. There is also evidence to suggest that heat shock proteins depend upon the activation of the mTOR pathway in order to be synthesized (Chou et al., 2012). If this is the case, this could be the reason why so few changes in HSP70 were observed. Chapter II presented results showing that two major downstream enzymes activated by the mTOR complex (4eBP1 and Rs6) were decreased in response to heat stress. This indicates that mTOR activity may have also been decreased, and potentially the activation of heat shock factor 1 as well.

**Table 3.1. Primers used in RT-PCR for skeletal muscle samples.**

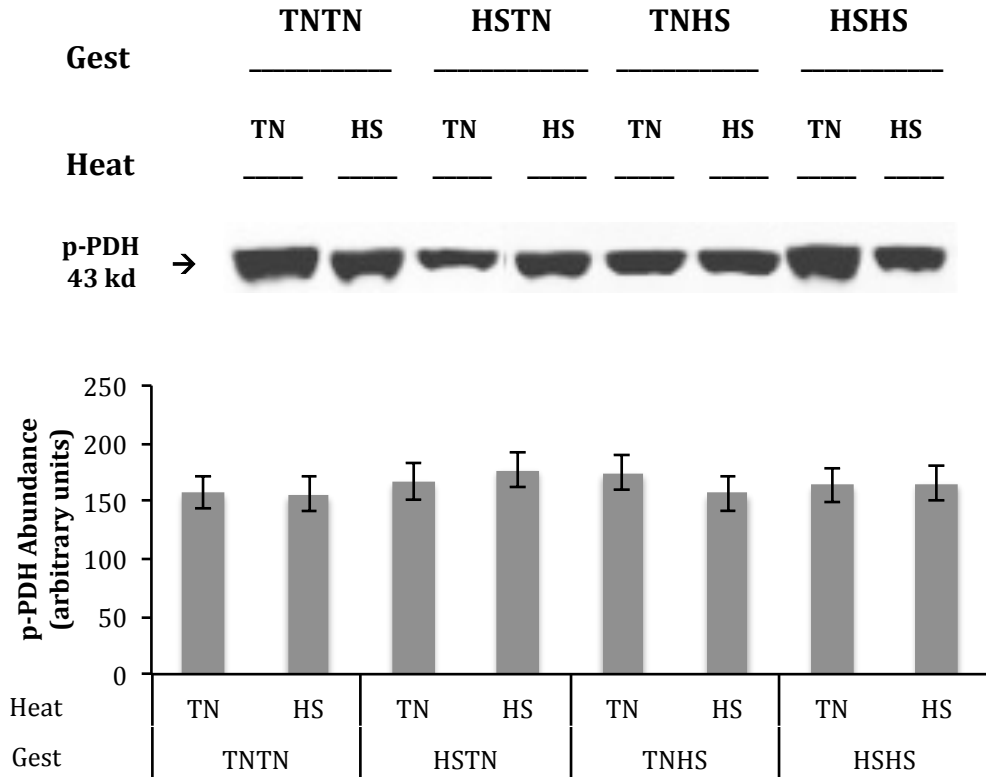
Gene	Direction	Sequence 5' to 3'
Atrogin1	Forward	GGCACCGATCACCCCTGCAC
Atrogin1	Reverse	AACCCTTCTGCGGTGCGGTC
Cox5B	Forward	AGCGGGCGTGATGGCTTCAA
Cox5B	Reverse	CCGCACAACGGCGACTCCAT
CytB	Forward	CCGGATCCAACAACCCTACCGGA
CytB	Reverse	TGGGGTGGGGTGTTTAGTGGGT
HK2	Forward	CTCCGGATGGGACAGAACACGGA
HK2	Reverse	GTGGTCGAACAGCTGGGTGCC
Hsp70	Forward	AGCACAAGAAGGACATCAGC
Hsp70	Reverse	GAAGTCGATGCCCTCGAACA
MURF	Forward	CTCAGTGTCCATGTCTGGAGGCCGTT
MURF	Reverse	GGCCGACTGGAGCACTCCTGTTTGTA
ND1	Forward	GCAGCCGGACCTTTCGCCAT
ND1	Reverse	TGGTGTGTGTGGGTCGTGGGA
PDK4	Forward	CTGGGAGCACCACCCACCT
PDK4	Reverse	GCGCATCACAAAGCGAGCCG
PGC-1 $\alpha$	Forward	GAGATTCCGTATCACCACC
PGC-1 $\alpha$	Reverse	CTTTCAGACTCCCGCTTC
SDHA	Forward	TGTACGCCTGCGGAGAGGCT
SDHA	Reverse	ATGCCCGGCCGAAGACAACG
TFAM	Forward	CGCGAGTGGCGGGCATGATA
TFAM	Reverse	GCCCCGGAGAAGCGCCATTG



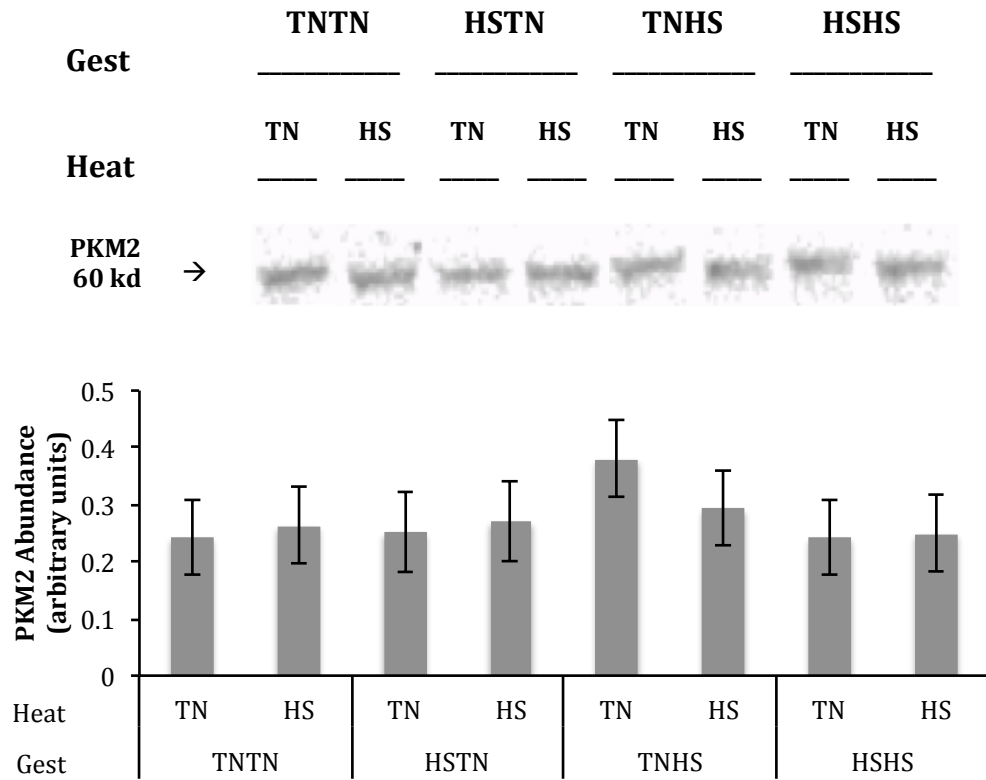
**Figure 3.1. Effects of acute heat stress on abundance of total GAPDH in skeletal muscle.** Top: Representative Western blot analysis of protein isolated from swine longissimus dorsi samples. Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 24 hours at 12 weeks of age. Bottom: Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



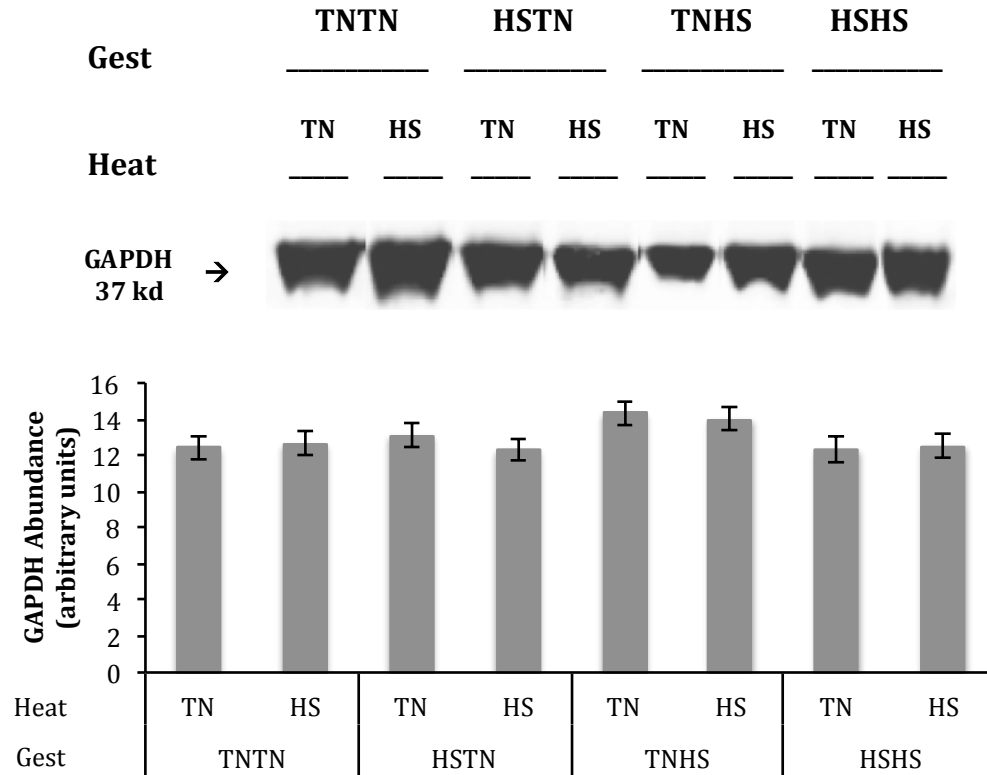
**Figure 3.2. Effects of acute heat stress on abundance of total PDH in skeletal muscle.** Top: Representative Western blot analysis of protein isolated from swine longissimus dorsi samples. Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 24 hours at 12 weeks of age. Bottom: Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



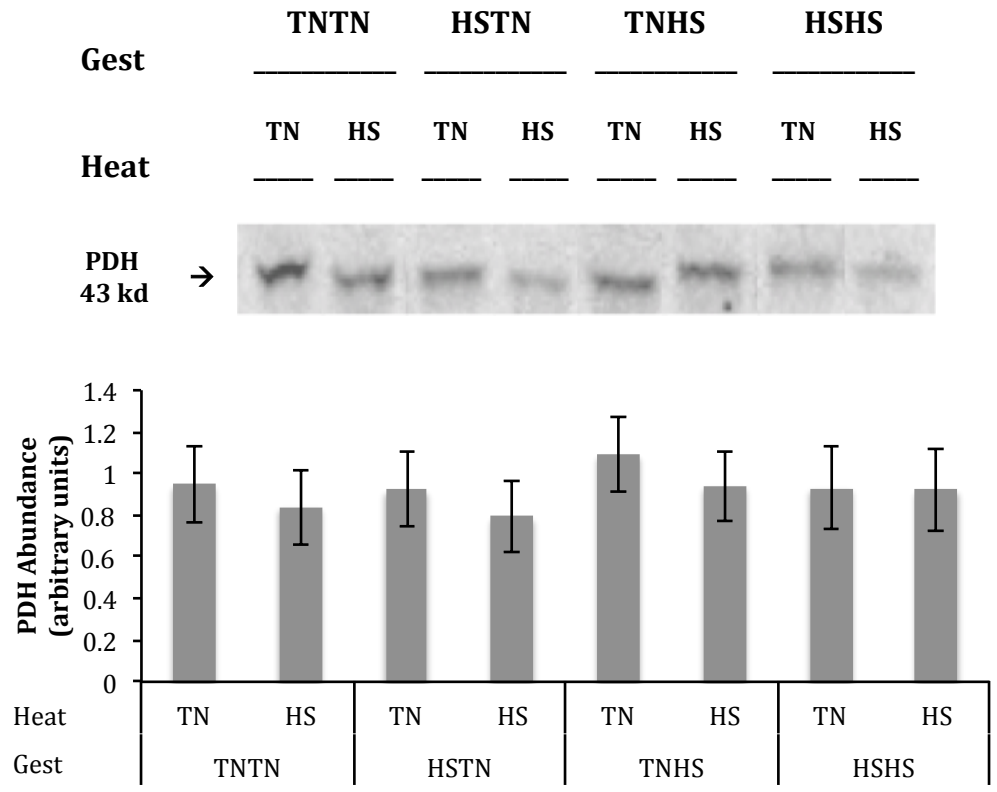
**Figure 3.3. Effects of acute heat stress on abundance of phosphorylated PDH in skeletal muscle.** Top: Representative Western blot analysis of protein isolated from swine longissimus dorsi samples. Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 24 hours at 12 weeks of age. Bottom: Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



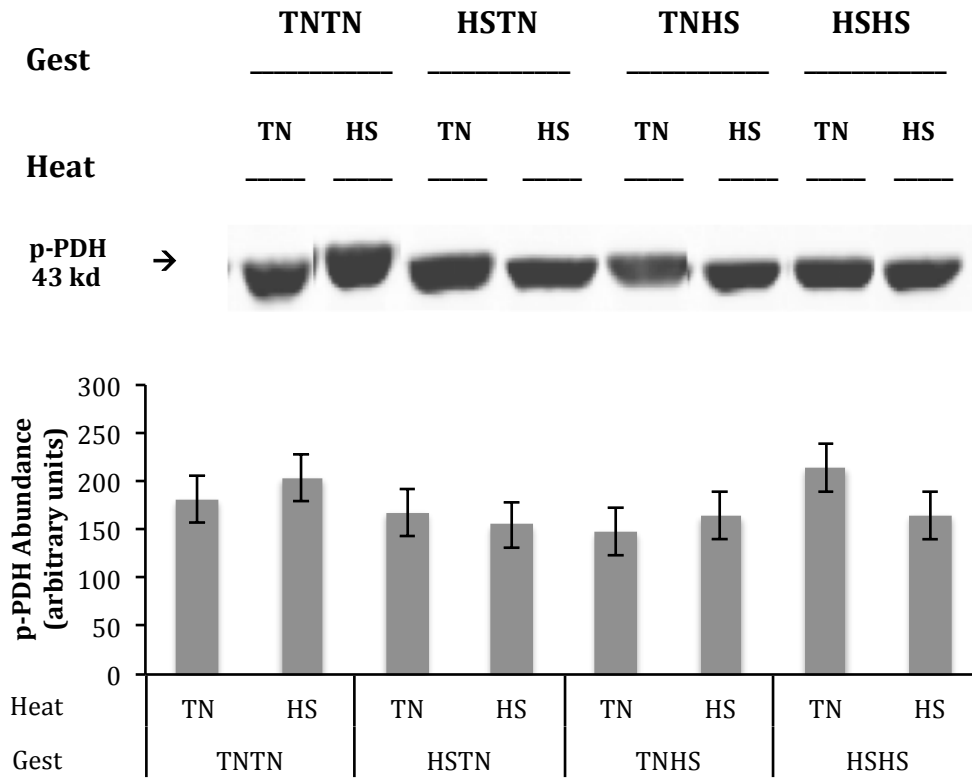
**Figure 3.4. Effects of acute heat stress on abundance of total PKM2 in skeletal muscle.** Top: Representative Western blot analysis of protein isolated from swine longissimus dorsi samples. Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 24 hours at 12 weeks of age. Bottom: Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



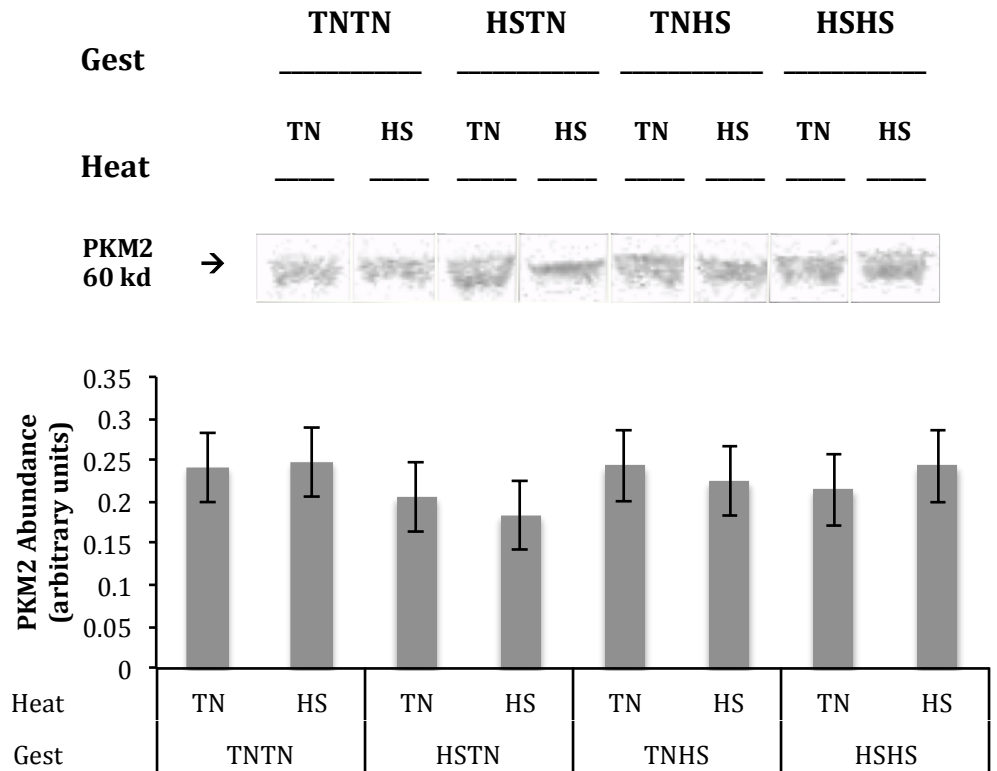
**Figure 3.5. Effects of chronic heat stress on abundance of total GAPDH in skeletal muscle.** Top: Representative Western blot analysis of protein isolated from swine longissimus dorsi samples. Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Bottom: Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



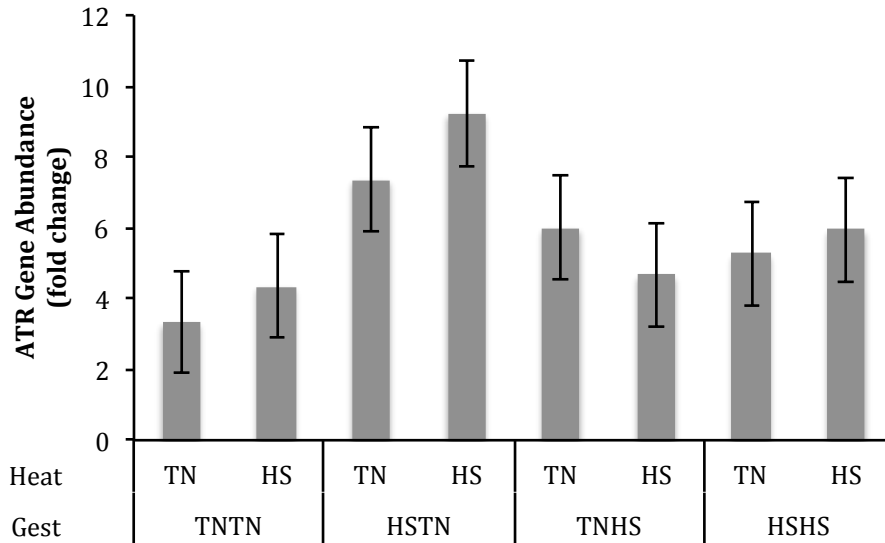
**Figure 3.6. Effects of chronic heat stress on abundance of total PDH in skeletal muscle.** Top: Representative Western blot analysis of protein isolated from swine longissimus dorsi samples. Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Bottom: Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



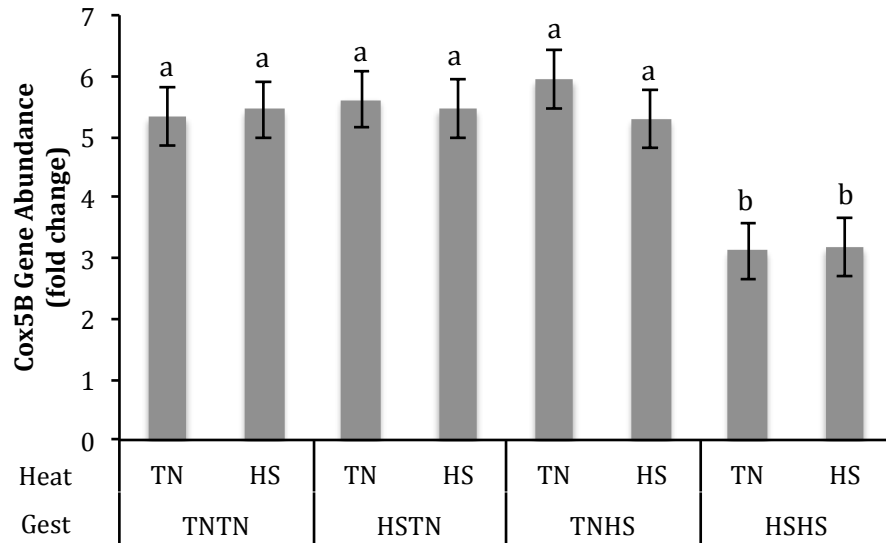
**Figure 3.7. Effects of chronic heat stress on abundance of phosphorylated PDH in skeletal muscle.** Top: Representative Western blot analysis of protein isolated from swine longissimus dorsi samples. Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Bottom: Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



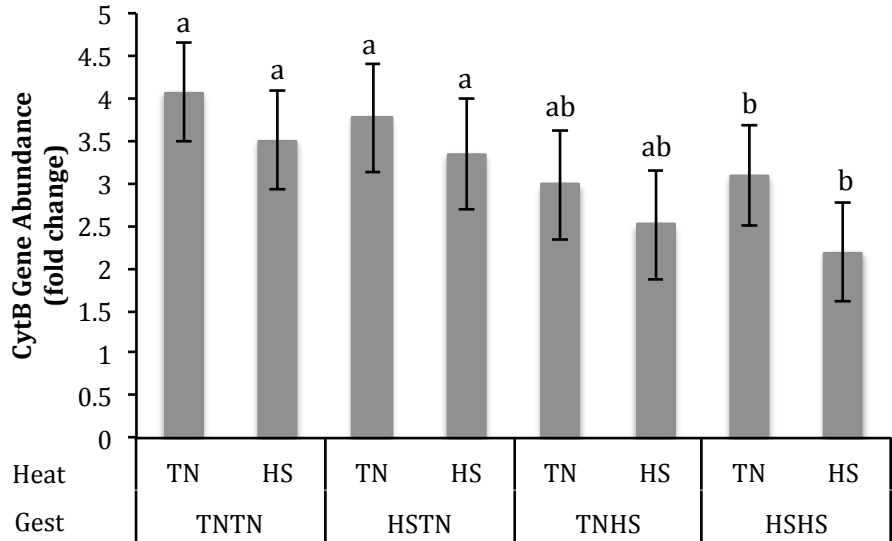
**Figure 3.8. Effects of chronic heat stress on abundance of total PKM2 in skeletal muscle.** Top: Representative Western blot analysis of protein isolated from swine longissimus dorsi samples. Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Bottom: Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



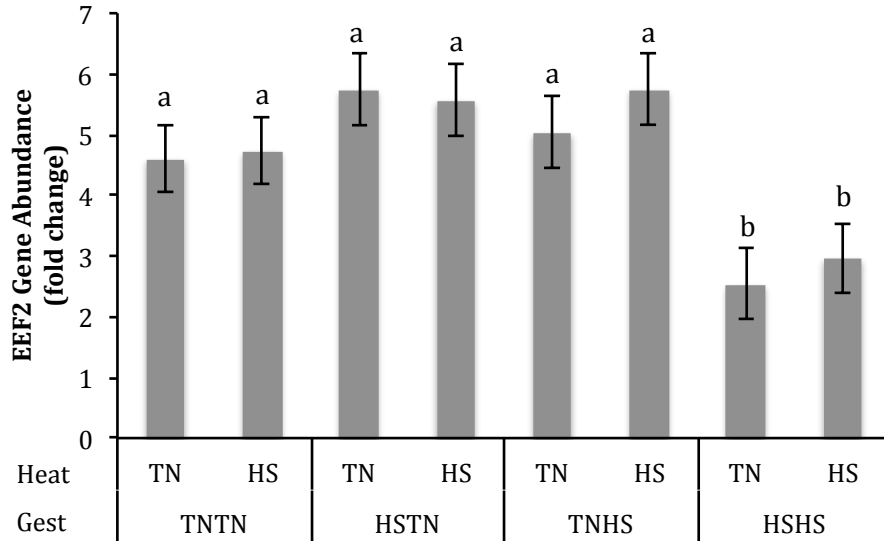
**Figure 3.9. Effects of acute heat stress on gene expression of ATR in skeletal muscle.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Total RNA was isolated from swine longissimus dorsi samples. Chart is a representation of data obtained through Real Time PCR. Fold change for each RNA sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



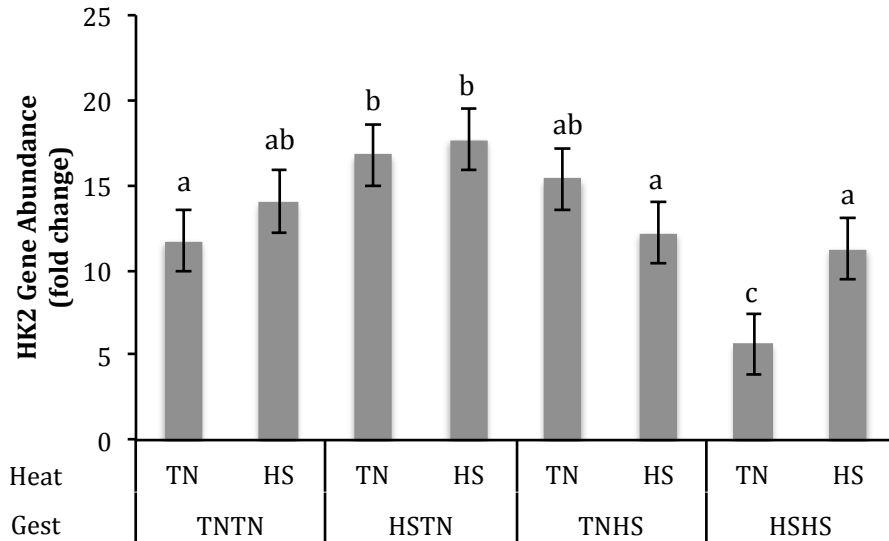
**Figure 3.10. Effects of acute heat stress on gene expression of Cox5B in skeletal muscle.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Total RNA was isolated from swine longissimus dorsi samples. Chart is a representation of data obtained through Real Time PCR. Fold change for each RNA sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis. Different letters symbolize significant treatment differences ( $p < 0.05$ ).



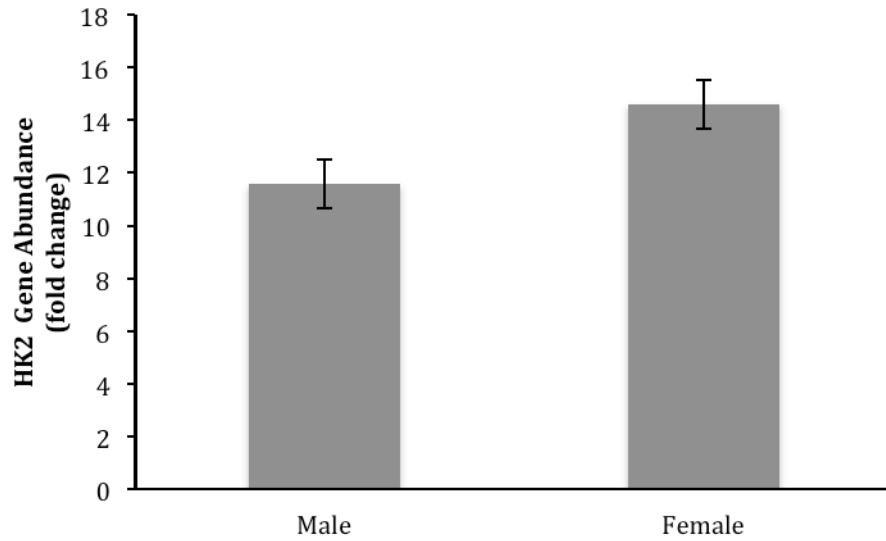
**Figure 3.11. Effects of acute heat stress on gene expression of CytB in skeletal muscle.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Total RNA was isolated from swine longissimus dorsi samples. Chart is a representation of data obtained through Real Time PCR. Fold change for each RNA sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis. Different letters symbolize significant treatment differences ( $p < 0.05$ ).



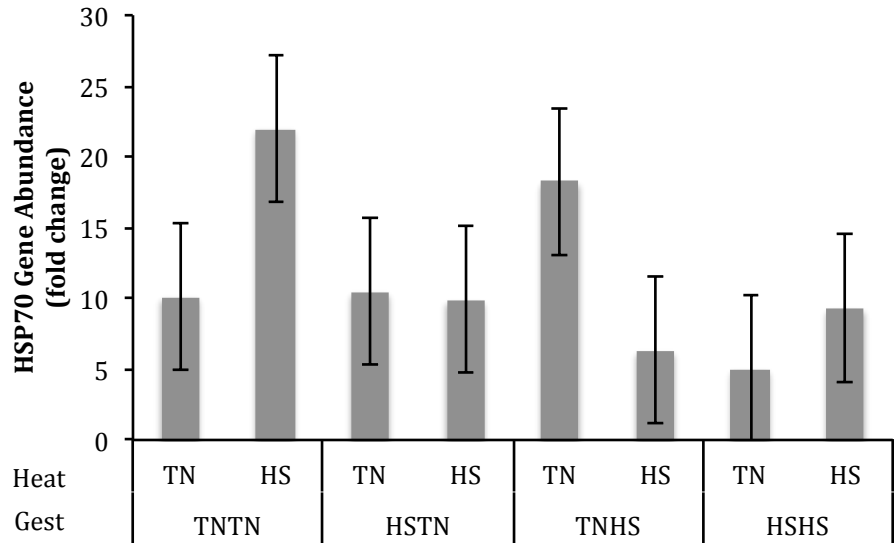
**Figure 3.12. Effects of acute heat stress on gene expression of EEF2 in skeletal muscle.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Total RNA was isolated from swine longissimus dorsi samples. Chart is a representation of data obtained through Real Time PCR. Fold change for each RNA sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis. Different letters symbolize significant treatment differences ( $p < 0.05$ ).



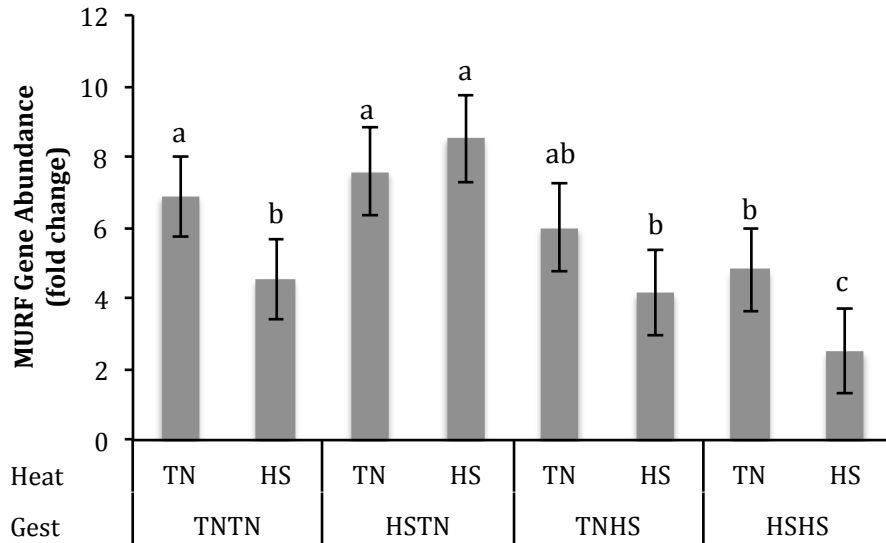
**Figure 3.13. Effects of acute heat stress on gene expression of HK2 in skeletal muscle.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Total RNA was isolated from swine longissimus dorsi samples. Chart is a representation of data obtained through Real Time PCR. Fold change for each RNA sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis. Different letters symbolize significant treatment differences ( $p < 0.05$ ).



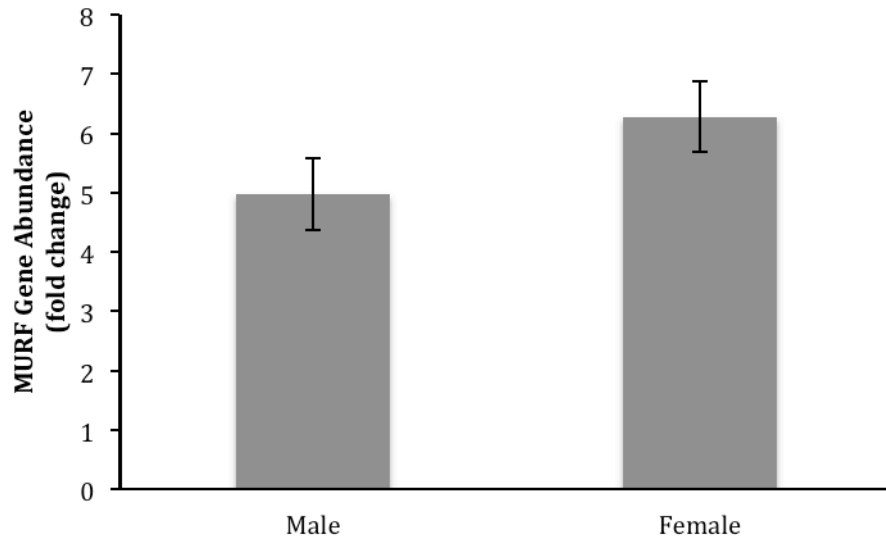
**Figure 3.14. Effects of sex on HK2 gene expression in swine under acute heat stress.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 24 hours at 12 weeks of age. Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis. Sex differences were found to be significant at  $p < 0.05$ .



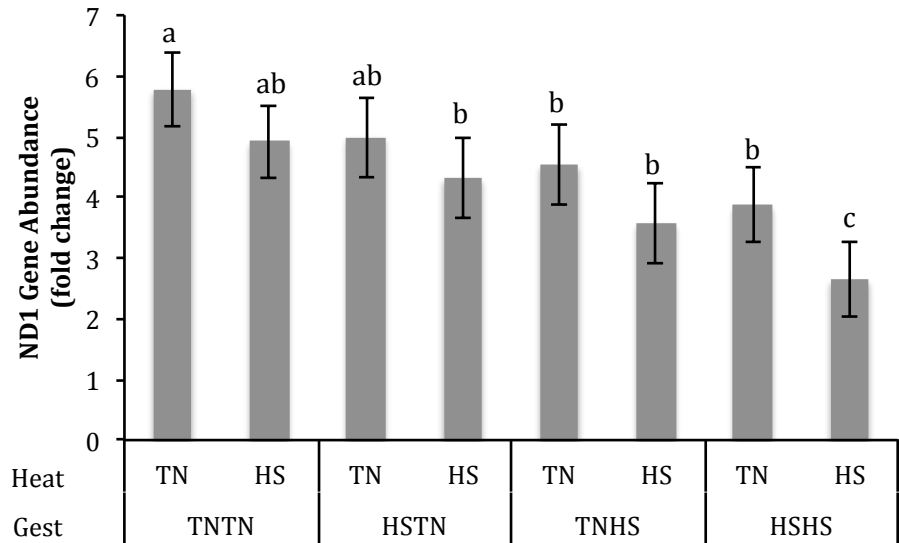
**Figure 3.15. Effects of acute heat stress on gene expression of HSP70 in skeletal muscle.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Total RNA was isolated from swine longissimus dorsi samples. Chart is a representation of data obtained through Real Time PCR. Fold change for each RNA sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



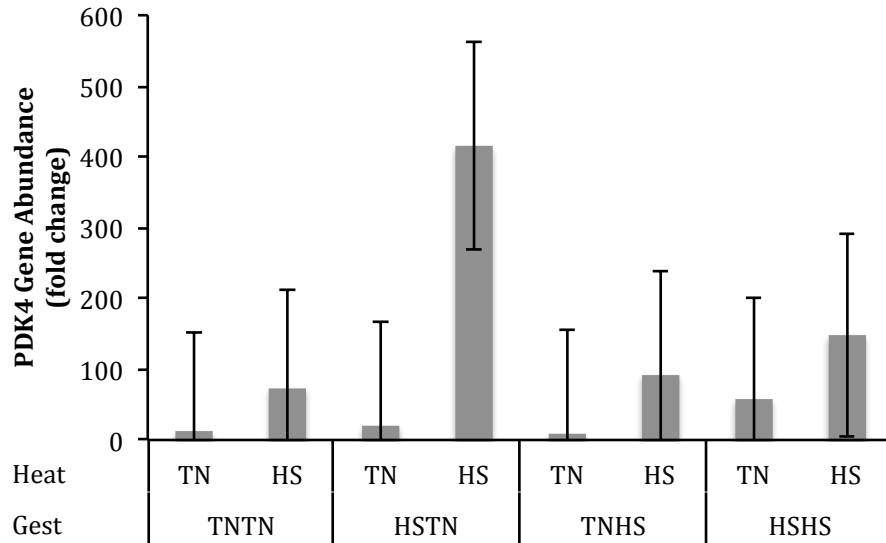
**Figure 3.16. Effects of acute heat stress on gene expression of MURF in skeletal muscle.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Total RNA was isolated from swine longissimus dorsi samples. Chart is a representation of data obtained through Real Time PCR. Fold change for each RNA sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis. Different letters symbolize significant treatment differences ( $p < 0.05$ ).



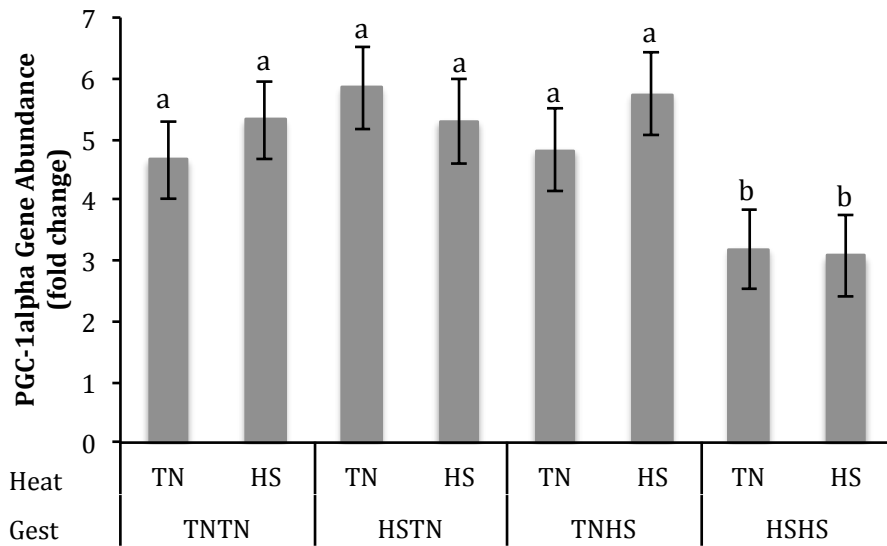
**Figure 3.17. Effects of sex on MURF gene expression in swine under acute heat stress.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 24 hours at 12 weeks of age. Raw abundance for each protein sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis. Sex differences were found to be significant at  $p < 0.05$ .



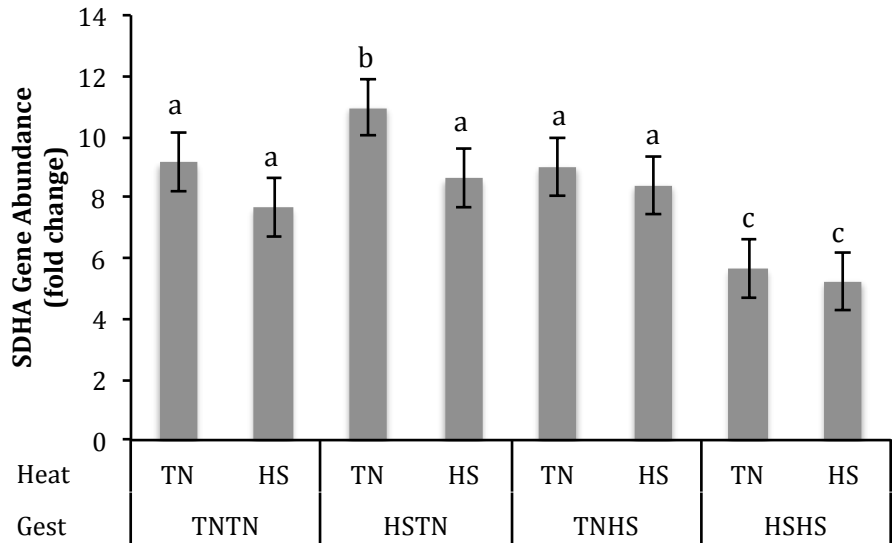
**Figure 3.18. Effects of acute heat stress on gene expression of ND1 in skeletal muscle.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Total RNA was isolated from swine longissimus dorsi samples. Chart is a representation of data obtained through Real Time PCR. Fold change for each RNA sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis. Different letters symbolize significant treatment differences ( $p < 0.05$ ).



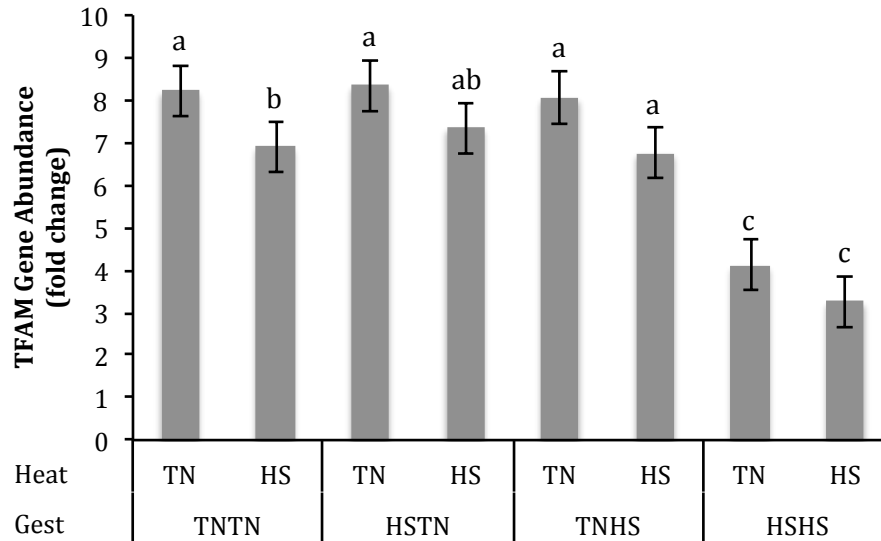
**Figure 3.19. Effects of acute heat stress on gene expression of PDK4 in skeletal muscle.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Total RNA was isolated from swine longissimus dorsi samples. Chart is a representation of data obtained through Real Time PCR. Fold change for each RNA sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



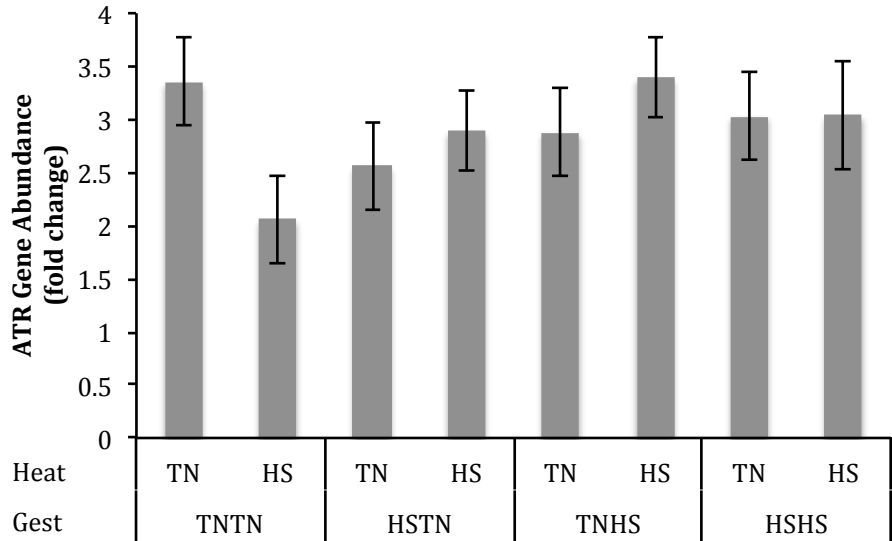
**Figure 3.20. Effects of acute heat stress on gene expression of PGC-1 $\alpha$  in skeletal muscle.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Total RNA was isolated from swine longissimus dorsi samples. Chart is a representation of data obtained through Real Time PCR. Fold change for each RNA sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis. Different letters symbolize significant treatment differences ( $p < 0.05$ ).



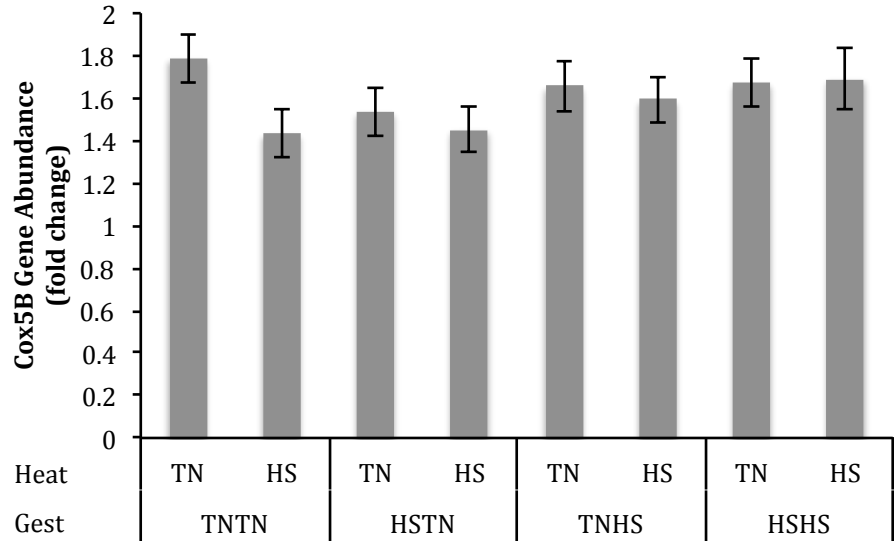
**Figure 3.21. Effects of acute heat stress on gene expression of SDHA in skeletal muscle.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Total RNA was isolated from swine longissimus dorsi samples. Chart is a representation of data obtained through Real Time PCR. Fold change for each RNA sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis. Different letters symbolize significant treatment differences ( $p < 0.05$ ).



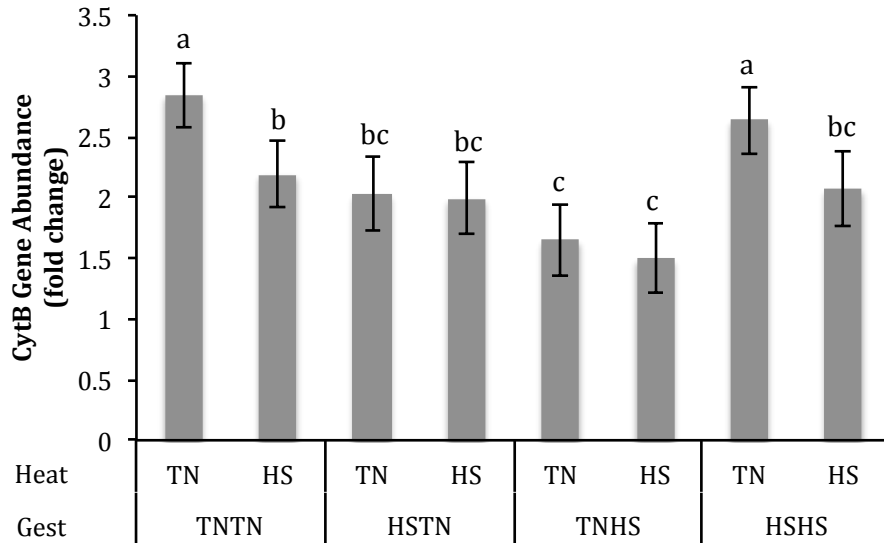
**Figure 3.22. Effects of acute heat stress on gene expression of TFAM in skeletal muscle.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Total RNA was isolated from swine longissimus dorsi samples. Chart is a representation of data obtained through Real Time PCR. Fold change for each RNA sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis. Different letters symbolize significant treatment differences ( $p < 0.05$ ).



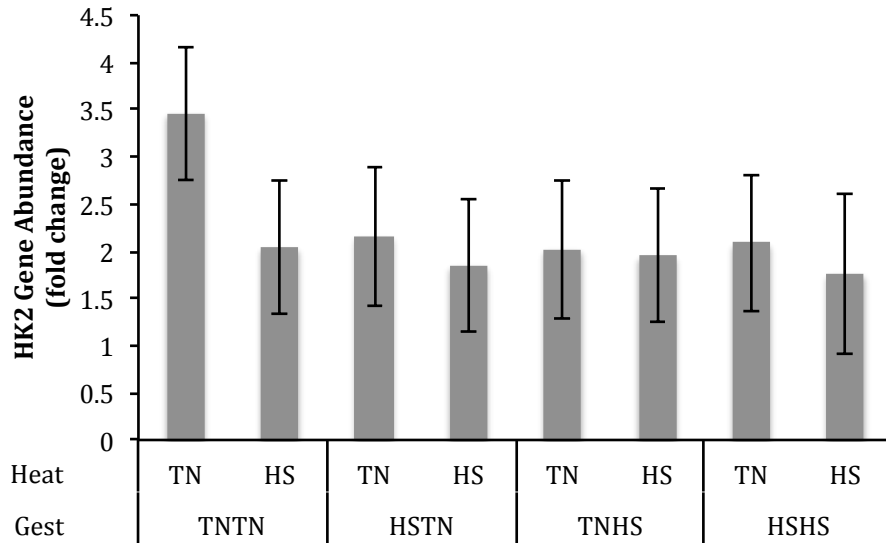
**Figure 3.23. Effects of chronic heat stress on gene expression of ATR in skeletal muscle.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Total RNA was isolated from swine longissimus dorsi samples. Chart is a representation of data obtained through Real Time PCR. Fold change for each RNA sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



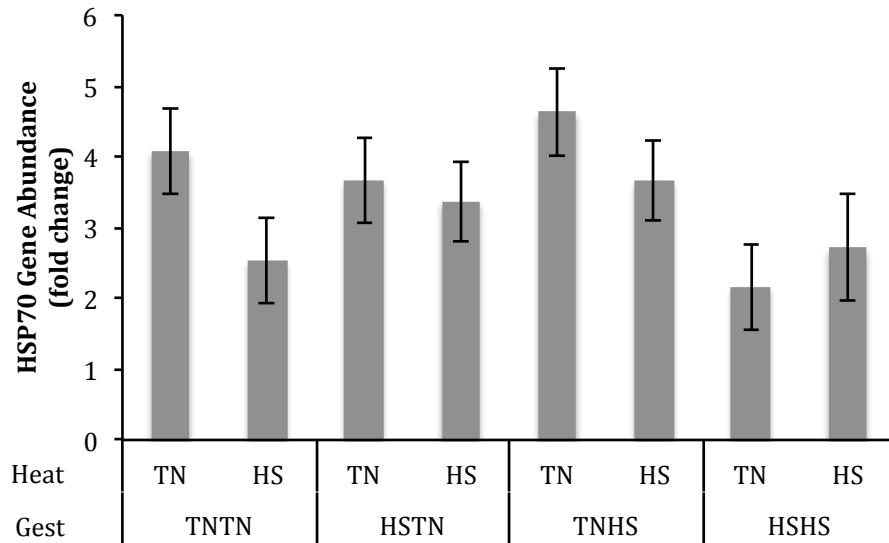
**Figure 3.24. Effects of chronic heat stress on gene expression of Cox5B in skeletal muscle.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Total RNA was isolated from swine longissimus dorsi samples. Chart is a representation of data obtained through Real Time PCR. Fold change for each RNA sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



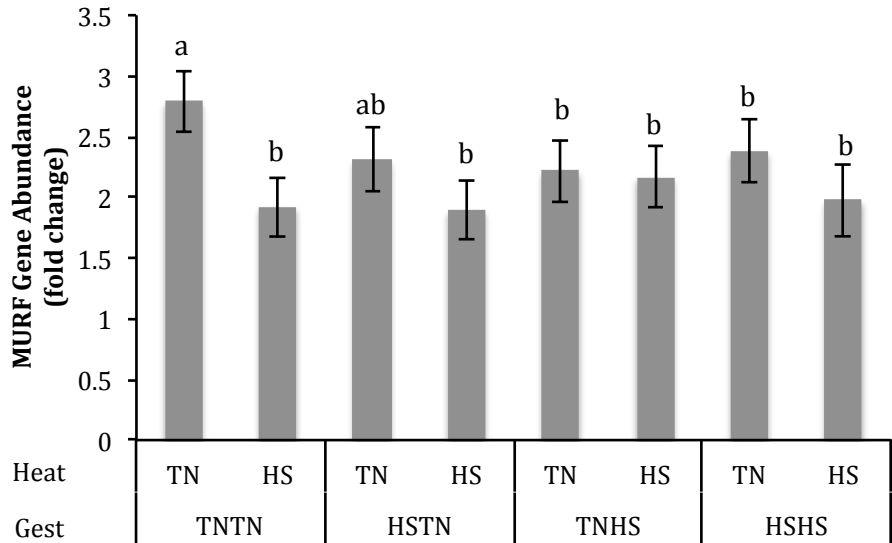
**Figure 3.25. Effects of chronic heat stress on gene expression of CytB in skeletal muscle.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Total RNA was isolated from swine longissimus dorsi samples. Chart is a representation of data obtained through Real Time PCR. Fold change for each RNA sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis. Different letters symbolize significant treatment differences ( $p < 0.05$ ).



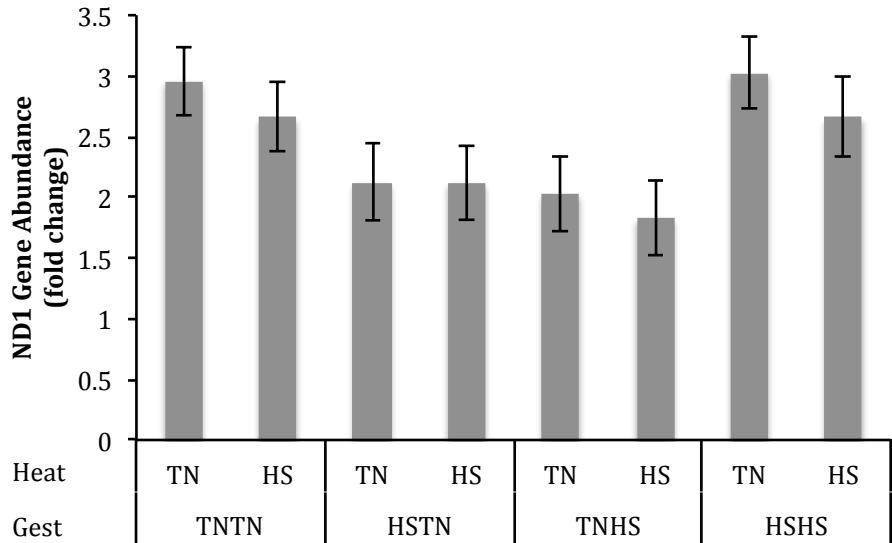
**Figure 3.26. Effects of chronic heat stress on gene expression of HK2 in skeletal muscle.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Total RNA was isolated from swine longissimus dorsi samples. Chart is a representation of data obtained through Real Time PCR. Fold change for each RNA sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



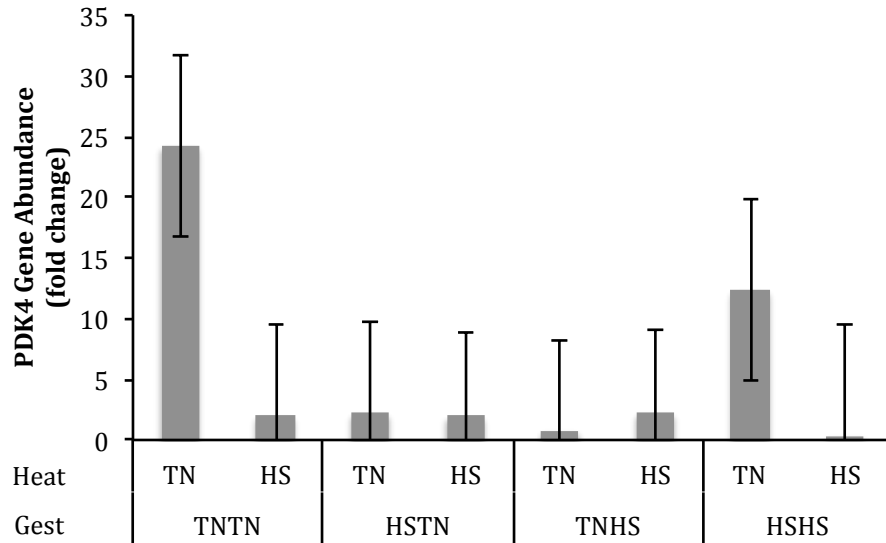
**Figure 3.27. Effects of chronic heat stress on gene expression of HSP70 in skeletal muscle.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Total RNA was isolated from swine longissimus dorsi samples. Chart is a representation of data obtained through Real Time PCR. Fold change for each RNA sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



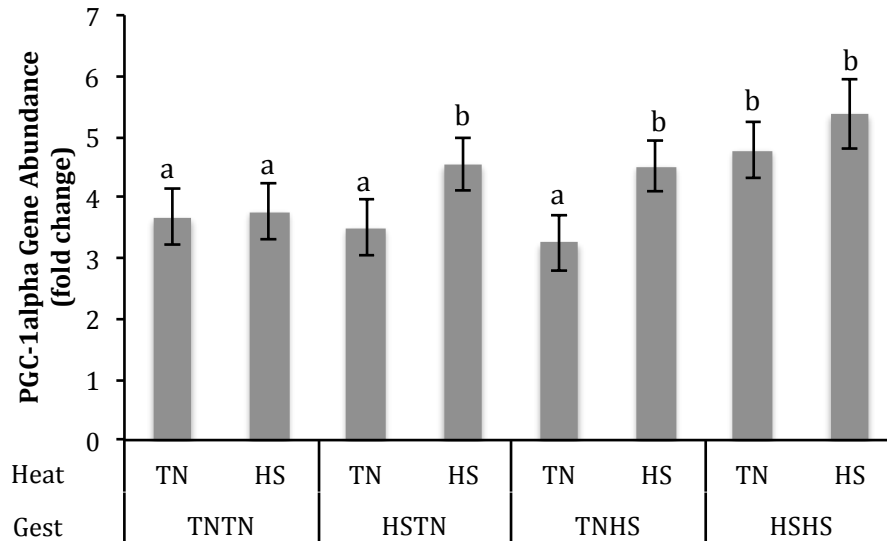
**Figure 3.28. Effects of chronic heat stress on gene expression of MURF in skeletal muscle.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Total RNA was isolated from swine longissimus dorsi samples. Chart is a representation of data obtained through Real Time PCR. Fold change for each RNA sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis. Different letters symbolize significant treatment differences ( $p < 0.05$ ).



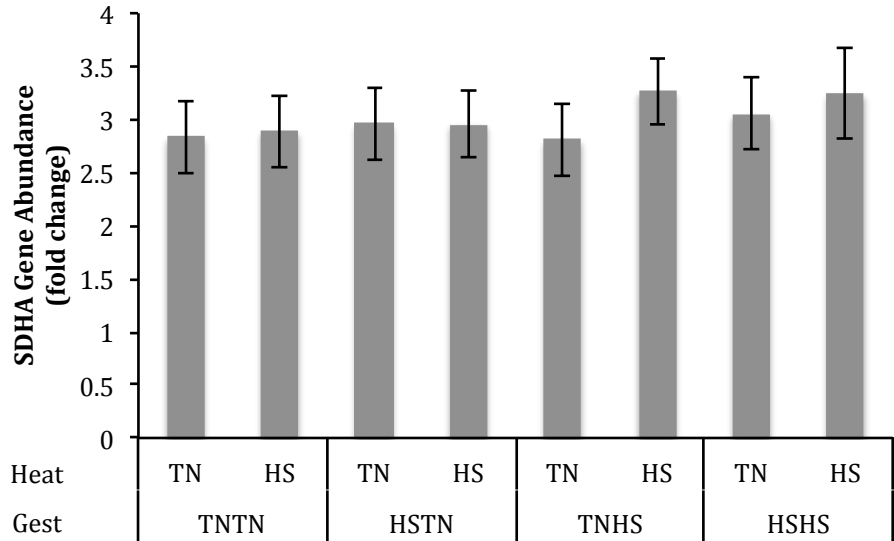
**Figure 3.29. Effects of chronic heat stress on gene expression of ND1 in skeletal muscle.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Total RNA was isolated from swine longissimus dorsi samples. Chart is a representation of data obtained through Real Time PCR. Fold change for each RNA sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



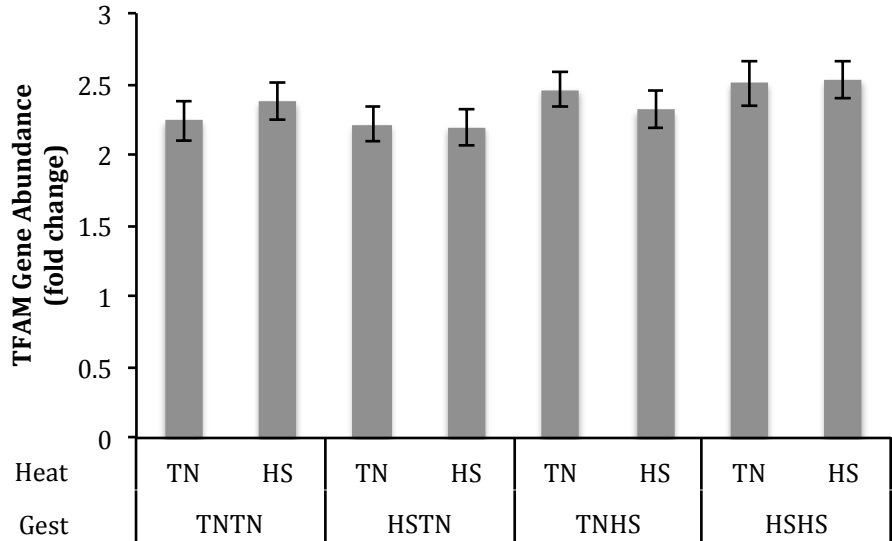
**Figure 3.30. Effects of chronic heat stress on gene expression of PDK4 in skeletal muscle.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Total RNA was isolated from swine longissimus dorsi samples. Chart is a representation of data obtained through Real Time PCR. Fold change for each RNA sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



**Figure 3.31. Effects of chronic heat stress on gene expression of PGC-1 $\alpha$  in skeletal muscle.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Total RNA was isolated from swine longissimus dorsi samples. Chart is a representation of data obtained through Real Time PCR. Fold change for each RNA sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis. Different letters symbolize significant treatment differences ( $p < 0.05$ ).



**Figure 3.32. Effects of chronic heat stress on gene expression of SDHA in skeletal muscle.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Total RNA was isolated from swine longissimus dorsi samples. Chart is a representation of data obtained through Real Time PCR. Fold change for each RNA sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.



**Figure 3.33. Effects of chronic heat stress on gene expression of TFAM in skeletal muscle.** Sows were prenatally exposed to 1 of 4 environmental treatments involving thermal neutral (TN, 25°C) or heat stress conditions (HS cyclical 28-34°C). Sows exposed to TN or HS throughout gestation are denoted TNTN and HSHS, respectively whereas sows heat-stressed for the first or second half of gestation are denoted HSTN and TNHS, respectively. Offspring were exposed to either constant TN (21°C) or HS (35°C) conditions for 5 weeks at 14 weeks of age. Total RNA was isolated from swine longissimus dorsi samples. Chart is a representation of data obtained through Real Time PCR. Fold change for each RNA sample was analyzed using the proc mixed procedure of SAS. A total of 48 animals were used for this analysis.

## Literature Cited

- Aarnink, A. J. A., J. W. Schrama, M. J. W. Heetkamp, J. Stefanowska, and T. T. T. Huynh. 2006. Temperature and body weight affect fouling of pig pens. *J. Anim. Sci.* 84:2224-2231.
- Aberle, E. D., R. A. Merkel, J. C. Forrest, and C. W. Alliston. 1974. Physiological responses of stress susceptible and stress resistant pigs to heat stress. *J. Anim. Sci.* 38:954-959.
- Abbot, E. L., J. G. McCormack, C. Reynet, D. G. Hassall, K. W. Buchan, and S. J. Yeaman. 2005. Diverging regulation of pyruvate dehydrogenase kinase isoform gene expression in cultured human muscle cells. *FEBS Journal.* 272:3004-3014.
- Angus, D.J., M.A. Febbraio, D. Lasini and M. Hargreaves. 2001. Effect of carbohydrate ingestion on glucose kinetics during exercise in the heat. *J. Appl. Physiol.* 90:601-605.
- Berg, J.M., J.L. Tymoczko and L. Stryer. 2007. *Biochemistry.* Freeman Custom Publishing, NY 6<sup>th</sup> ed.
- Boddicker, R. L., N. J. Boddicker, J. N. Rhoades, S. Pearce, J. Johnson, M. C. Lucy, T. J. Safranski, N. K. Gabler, J. T. Selsby, S. Lonergan, J. Patience, R. P. Rhoads, J. C. M. Dekkers, L. H. Baumgard, and J. W. Ross. 2012. Heat stress experienced in utero alters postnatal body composition parameters in growing pigs. *J. Anim. Sci. Vol. 90, E-Suppl. 1:* 401.
- Bornman L., C.M. Steinmann, G.S. Gericke and S. Polla. 1998. In vivo heat shock protects rat myocardial mitochondria. *Biochem. Biophys. Res. Commun.* 246:836-840.
- Burger, M., A. Lawen, and O. H. W. Martini. 1988. Insulin-induced S6 kinase activation in HeLa cells and its reversal by hyperthermic stress. *Eur. J. Biochem.* 183:255-262.
- Campbell, M.K. 1999. *Biochemistry.* Saunders College Publishing, NY. 3<sup>rd</sup> ed.
- Campbell R. G. and M. R. Taverner. 1988. Relationships between energy intake and protein and energy metabolism, growth and body composition of pigs kept at 14 or 32°C from 9 to 20 kg. *Livest. Prod. Sci.* 18:289-303.
- Caspani, M.L., M. Savioli, S. Crotti, P. Bruzzone and L. Gattinoni. 2004. Heat stress characteristics, pathophysiology and avoidable mistakes. *Minerva Anesthesiol.* 70:617-624.
- Chou, S., T. Prince, J. Gong, and S. K. Calderwood. 2012. mTOR is essential for the proteotoxic stress response, HSF1 activation and heat shock protein synthesis. *PLoS1* 7:e39679.

- Collin, A., J. van Milgen, S. Dubois, and J. Noblet. 2001. Effect of high temperature on feeding behavior and heat production in group-housed young pigs. *Brit. J. Nutr.* 86:63-70.
- Collin, A., J. van Milgen, S. Dubois, and J. Noblet. 2001. Effect of high temperature and feeding level on energy utilization in piglets. *J. Anim. Sci.* 79:1849-1857.
- Dann, S. G., and G. Thomas. 2006. The amino acid sensitive TOR pathway from yeast to mammals. *FEBS Letters.* 580:2821-2829.
- Davidson, J.F. and R.H. Schiestl. 2001. Mitochondrial respiratory electron carriers are involved in oxidative stress during heat stress in *S. cerevisiae*. *Mol. Cell Biol.* 21: 8483-8489.
- Duncan, R. F. and J. W. B. Hershey. 1989. Protein synthesis and protein phosphorylation during heat stress, recovery, and adaptation. *J. Cell. Biol.* 108:1467-1481.
- Febbraio, M.A., R.J. Snow, M. Hargreaves, C.G. Stathis, I.K. Martin and M.F. Carey. 1994a. Muscle metabolism during exercise and heat stress in trained men: effect of acclimation. *J. Appl. Physiol.* 76:589-97.
- Febbraio, M.A., R.J. Snow, C.G. Stathis, M. Hargreaves and M.F. Carey. 1994b. Effect of heat stress on muscle energy metabolism during exercise. *J. Appl. Physiol.* 77:2827-2831.
- Febbraio, M.A., R.J. Snow, C.G. Stathis and M. Hargreaves. 1996. Blunting the rise in body temperature reduced muscle glycogenolysis during exercise in humans. *Exp. Physiol.* 81:685-693.
- Febbraio, M. A. 2001. Alterations in energy metabolism during exercise and heat stress. *Sports Med.* 31:47-59.
- Fink, W., D. Costill, and P. Van Handel. 1975. Leg muscle metabolism during exercise in the heat and cold. *Euro. J. Appl. Physiol. Occup. Physiol.* 34:183-190.
- Frier, B. and M. Locke. 2007. Heat stress inhibits skeletal muscle hypertrophy. *Cell Stress Chaperon.* 12:132-141.
- Fuquay, J. W. 1981. Heat stress as it affects animal production. *J. Anim. Sci.* 52:164-174.
- Geraert, P. A., J. C. F. Padilha, and S. Guillaumin. 1996. Metabolic and endocrine changes induced by chronic heat exposure in broiler chickens: growth performance, body composition and energy retention. *Brit. J. Nutr.* 75:195-204.
- Glass, D. J. 2003. Signalling pathways that mediate skeletal muscle hypertrophy and atrophy. *Nat. Cell Biol.* 5:87-90.

- Gonzalez-Alonso, J., J.A.L. Calbet, B. Nielsen. 1999. Metabolic and thermodynamic responses to dehydration-induced reductions in blood flow in exercising humans. *J. Physiol.* 520:577-589.
- Gudi, R., M. M. Bowker-Kinley, N. Y. Kedishvili, Y. Zhao, and K. M. Popov. 1995. Diversity of the pyruvate dehydrogenase kinase family in humans. *J. Biol. Chem.* 48:28989-28994.
- Hargreaves, M., D. Angus, K. Howlett, N. Marmy-Conus and M. Febbraio. 1996. Effect of heat stress on glucose kinetics during exercise. *J. Appl. Physiol.* 81:1594-1597.
- Heise, K., S. Puntamlo, H.O. Portner and D. Abele. 2003. Production of reactive oxygen species by isolated mitochondria of the Antarctic bivalve *Laternula elliptica* under heat stress. *Comp. Biochem. Physiol. Toxicol. Pharmacol.* 134:79-90.
- Henle, K.J. and D.B. Leeper. 1979. Effects of hyperthermia on macromolecular synthesis in Chinese hamster ovary cells. *Cancer Res.* 39:2665-2674.
- Himms-Hagen, J. 1976. Cellular thermogenesis. *Annu. Rev. Physiol.* 38:315-351.
- Hood, D. A. 2009. Mechanisms of exercise-induced mitochondrial biogenesis in skeletal muscle. *Appl. Physiol. Nutr. Metab.* 34:465-472.
- Hsu, Y.D., S.S. Chen, W.H. Lee, S.Z. Lin, M.C. Kao and W.L. Tsao. 1995. Mitochondrial alterations of skeletal muscle in a heat stress rat model. *Proc. Natl. Sci. Council, ROC Part B Life Sci.* 19(4):233-239.
- Hubbard, R.W. 1990. Heatstroke pathophysiology: the energy depletion model. *Med. Sci. Sports Exerc.* 22:19-28.
- Huynh, T. T. T., A. J. A. Aarnink, M. W. S. Verstegen, W. J. J. Gerrits, M. J. W. Heetkamp, B. Kemp, and T. T. Canh. 2005. Effects of increasing temperature on physiological changes in pigs at different relative humidities. *J. Anim. Sci.* 83:1385-1396.
- Johnson, J. S., R. L. Boddicker, S. Pearce, M. V. Sanz-Fernandez, M. C. Lucy, T. J. Safranski, N. K. Gabler, R. P. Rhoads, J. W. Ross, J. Patience, S. Lonergan, L. H. Baumgard, and J. Selsby. 2012. Gestational thermal environment alters postnatal response to heat stress. *FASEB J.* 26:1079.4.
- Kamanga-Sollo, E., M. S. Pampusch, M. E. White, M R. Hathaway, and W. R. Dayton. 2011. Effects of heat stress on proliferation, protein turnover, and abundance of heat shock protein messenger ribonucleic acid in cultured porcine muscle satellite cells. *J. Anim. Sci.* 89:3473-3480.
- Kerr, B. J., J. T. Yen, J. A. Nienaber, and R. A. Easter. 2003. Influences of dietary protein level, amino acid supplementation and environmental temperature on

performance, body composition, organ weights and total heat production of growing pigs. *J. Anim. Sci.* 81:1998-2007.

Kimball, S. R. 1999. Eukaryotic initiation factor eIF2. *Int. J. Biochem. Cell B.* 31:25-29.

Koyama, S. S. Hata, C. C. Witt, Y. Ono, S. Lerche, K. Ojima, T. Chiba, N. Doi, F. Kitamura, K. Abe, S. H. Witt, V. Rybin, A. Gasch, T. Franz, S. Labeit, and H. Sorimachi. 2008. Muscle RING-finger protein-1 (MuRF1) as a connector of muscle energy metabolism and protein synthesis. *J. Mol. Biol.* 376:1224-1236.

Kozlowski, S., Z. Brzezinska, B. Kruk, H. Kaciuba-Uscilko, J. E. Greenleaf, and K. Nazar. 1985. Exercise hyperthermia as a factor limiting physical performance: temperature effect on muscle metabolism. *J. Appl. Physiol.* 59:766-773.

Kregel, K. C. 2002. Invited review: heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. *J. Appl. Physiol.* 92:2177-2186.

Le Bellego, L., J. van Milgen, and J. Noblet. 2002. Effect of high ambient temperature on protein and lipid deposition and energy utilization in growing pigs. *Anim. Sci.* 75:85-96.

Lee, F. N., L. Zhang, D. Zheng, W. S. Choi, and J. H. Youn. 2004. Insulin suppresses PDK-4 expression in skeletal muscle independently of plasma FFA. *Am. J. Physiol. Endocrinol. Metab.* 287:E69-E74.

Lee, F.S. and E.L. Scott. 1916. The action of temperature and humidity on the working power of muscles and on the sugar of the blood. *Am. J. Physiol.* 40:486-501.

Lindquist, S. 1986. The heat-shock response. *Ann. Rev. Biochem.* 55:1151-1191.

Marple, D. N., D. J. Jones, C. W. Alliston, and J. C. Forrest. 1974. Physiological and endocrinological changes in response to terminal heat stress in swine. *J. Anim. Sci.* 39:79-82.

Mondovi, B., A. Finazzi-Agro, G. Rotilio, R. Strom, G. Moricca and A. Rossi-Fanelli. 1969. The biochemical mechanism of selective heat sensitivity of cancer cells. *Eur. J. Cancer* 5:137-146.

Morimoto, R.I. 1998. Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes Dev.*, 12:3788-3796.

Moseley, P. L. 1997. Heat shock proteins and heat adaptation of the whole organism. *J. Appl. Physiol.* 83:1413-1417.

- Mujahid, A., Y. Yoshiki, Y. Akiba, and M. Toyomizu. 2005. Superoxide radical production in chicken skeletal muscle induced by acute heat stress. *Poultry Science*. 84:307-314.
- O'Brien, M. D., R. P. Rhoads, S. R. Sanders, G. C. Duff, and L. H. Baumgard. 2010. Metabolic adaptations to heat stress in growing cattle. *Domest. Anim. Endocrin.* 38:86-94.
- Parkin, J.M., M.F. Carey, S. Zhao and M.A. Febbraio. 1999. Effect of ambient temperature on human skeletal muscle metabolism during fatiguing submaximal exercise. *J Appl Physiol* 86:902-908.
- Patience, J. F., J. F. Umboh, R. K. Chaplin, and C. M. Nyachoti. 2005. Nutritional and physiological responses of growing pigs to a diurnal pattern of heat stress. *Livest. Prod. Sci.* 96:205-214.
- Polak, P. and M. N. Hall. 2009. mTOR and the control of whole body metabolism. *Curr. Opin. Cell. Biol.* 21:209-218.
- Polla, B.S., S. Kantengwa, D. Francois, S. Salvioli, C. Franceschi, C. Marsac and A. Cossarizza. 1996. Mitochondria are selective targets for the protective effects of heat shock against oxidative injury. *Proc. Natl. Acad. Sci. U.S.A.* 93:6458-6463.
- Qian, L., M.P. Wu, X.J. Chen and S.Q. Cheng. 1992. The changes of ATP content in ventricle myocytes of heat shocked rats and its mechanism. *Chin. J. Ind. Hyg. Occup. Dis.* 10:233-236.
- Qian, L., X. Song, H. Ren, J. Gong and S. Cheng. 2004. Mitochondrial mechanism of heat stress-induced injury in rat cardiomyocyte. *Cell Stress Chaperones* 9:281-293.
- Renaudeau, D., J. L. Gourdine, B. A. N. Silva, and J. Noblet. 2008. Nutritional routes to attenuate heat stress in pigs. *Proceedings of the Livestock and Global Climate Change Conference.* 134-138.
- Renaudeau, D., A. Collin, S. Yahav, V. de Basilio, J. L. Gourdine, and R. J. Collier. 2011. Adaptation to hot climate and strategies to alleviate heat stress in livestock production. *Animal.* 1-22.
- Renaudeau, D. J. L. Gourdine, and N. R. St-Pierre. 2011. A meta-analysis of the effects of high ambient temperature on growth performance of growing-finishing pigs. *J. Anim. Sci.* 89:2220-2230.
- Rhoads, R. P., A. J. La Noce, J. B. Wheelock, and L. H. Baumgard. 2011. Short communication: Alterations in expression of gluconeogenic genes during heat stress and exogenous bovine somatotropin administration. *J. Dairy Sci.* 94:1917-1921.

- Richter, W. W., K. D. Zang, and O. G. Issinger. 1983. Influence of hyperthermia on the phosphorylation of ribosomal protein S6 from human skin fibroblasts and meningioma cells. *FEBS Letters*. 153:262-266.
- Roti Roti, J.L. 2008. Cellular responses to hyperthermia (40-46°C): Cell killing and molecular events. *Int. J. Hyperthermia* 24:3-15.
- Rowell, L.B., G.L. Brengelmann, J.R. Blackmon, R.D. Twiss and F. Kusumi. 1968. Splanchnic blood flow and metabolism in heat-stressed man. *J. Appl. Physiol.* 24:475-484.
- Sengupta, S., T. R. Peterson, and D. M. Sabatini. 2010. Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. *Mol. Cell.* 40:310-322.
- Song, X.L., L.J. Qian, and F.Z. Li. 2000. Injury of heat stress on rat cardiomyocytes. *Chin. J. Appl. Physiol.* 16:227-230.
- Sonna, L.A., S.L. Gaffin, R.E. Pratt, M.L. Cullivan, K.C. Angel and C.M. Lilly. 2002. Effect of acute heat shock on gene expression by human peripheral blood mononuclear cells. *J. Appl. Physiol.* 92:2208-2220.
- Spencer, J. D., R. D. Boyd, R. Cabrera, and G. L. Allee. 2003. Early weaning to reduce tissue mobilization in lactation sows and milk supplementation to enhance pig weaning weight during extreme heat stress. *J. Anim. Sci.* 81:2041-2052.
- St. Pierre, N. R., B. Cobanov, and G. Schnitkey. 2003. Economic losses from heat stress by US livestock industries. *J. Dairy Sci.* 86:E52-E77.
- Streffer, C. 1988. Aspects of metabolic change after hyperthermia. *Recent Results in Cancer Res.* 107:7-16.
- Sugden, M. C. and M. J. Holness. 2003. Recent advances in mechanisms regulating glucose oxidation at the level of the pyruvate dehydrogenase complex by PDKs. *Am. J. Physiol. Endocrinol. Metab.* 284:E855-E862.
- Sugden, M. C. and M. J. Holness. 2006. Mechanisms underlying regulation of the expression and activities of the mammalian pyruvate dehydrogenase kinases. *Arch. Physiol. and Biochem.* 112:139-149.
- Tas, P. W. L. and H. W. Martini. 1987. Regulation of ribosomal protein S6 phosphorylation in heat-shocked HeLa cells. *Eur. J. Biochem.* 163:553-559.
- Wenz, T., F. Diaz, B. M. Spiegelman, and C. T. Moraes. 2008. Activation of the PPAR/PGC-1 $\alpha$  pathway prevents a bioenergetics deficit and effectively improves a mitochondrial myopathy phenotype. *Cell Metab.* 8:249-256.

Wettemann, R. P and F. W. Bazer. 1985. Influence of environmental temperature on prolificacy of pigs. Abstract. J. Rep. Fer. S. 33:199-208.

Wullschleger, S., R. Loewith, and M. N. Hall. 2006. TOR signaling in growth and metabolism. Cell. 124:471-484.

Young, A.J., M.N. Sawka, L. Levine, B.S. Caderette and K.B. Pandolf. 1985. Skeletal muscle metabolism during exercise is influenced by heat acclimation. J. Appl. Physiol. 59:1929-1935.

## Appendix A

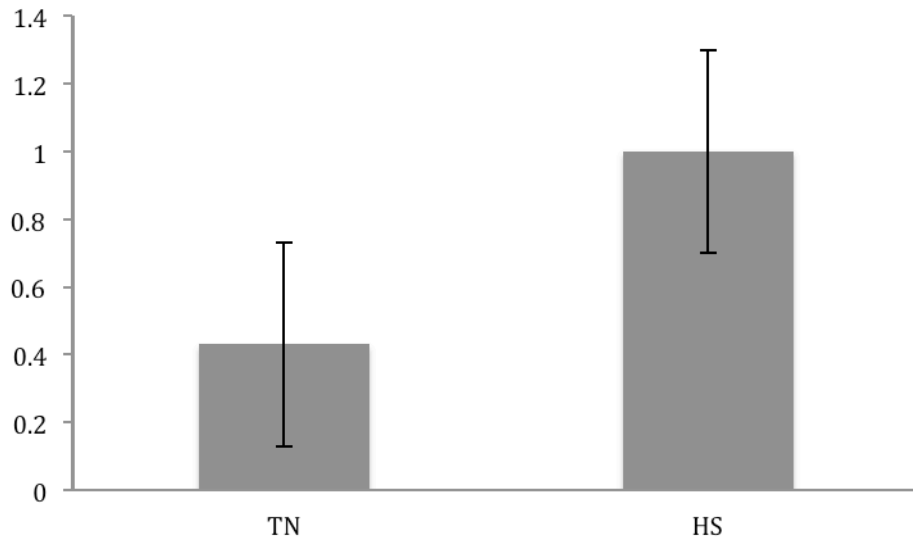


Figure A.1 Skin temperature ( $T_s$ ) and rectal temperature ( $T_r$ ) ratio of TN and HS gestational groups during acute heat stress. The HS gestational treatment group has a greater  $T_s$  to  $T_r$  difference by ~50% (adapted from Johnson et al., 2012).

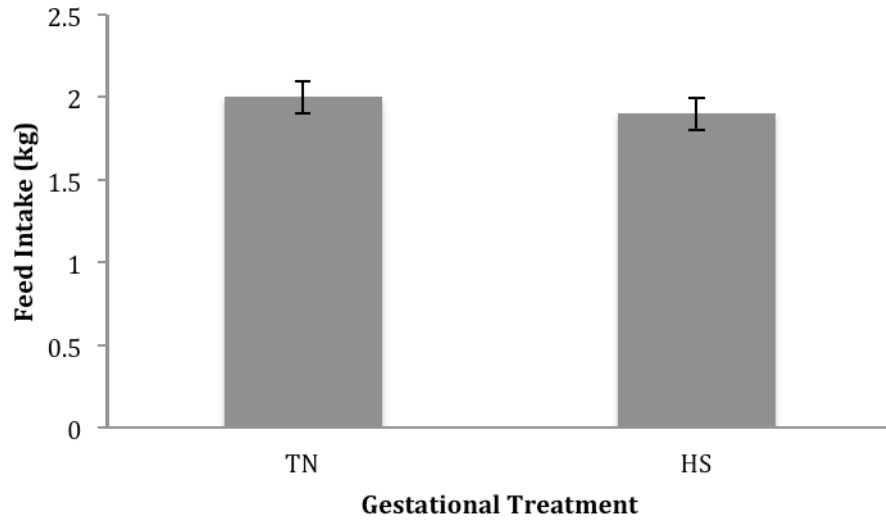


Figure A.2 Feed intake during 24 h acute heat stress. No differences occurred ( $p = 0.7166$ ; adapted from Johnson et al., 2012).

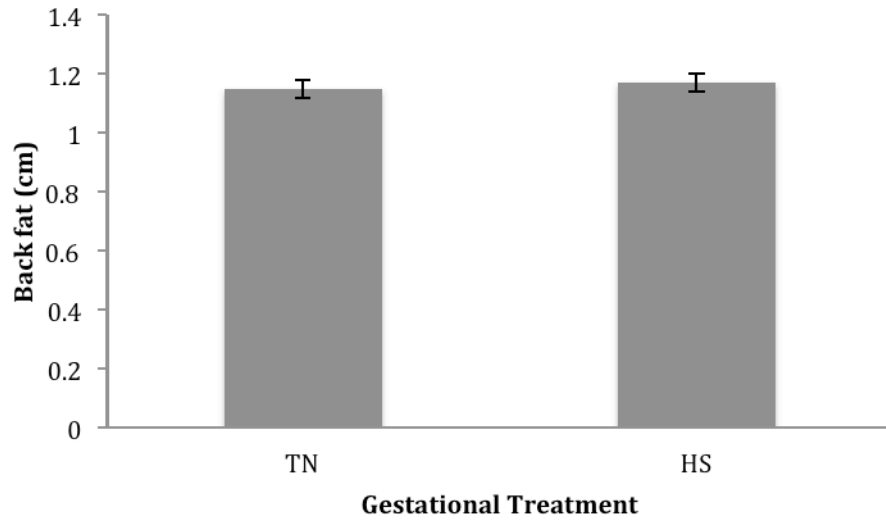


Figure A.3 Back fat (cm) of TN and HS gestational groups. No differences occurred ( $p = 0.3929$ ; adapted from Johnson et al., 2012).

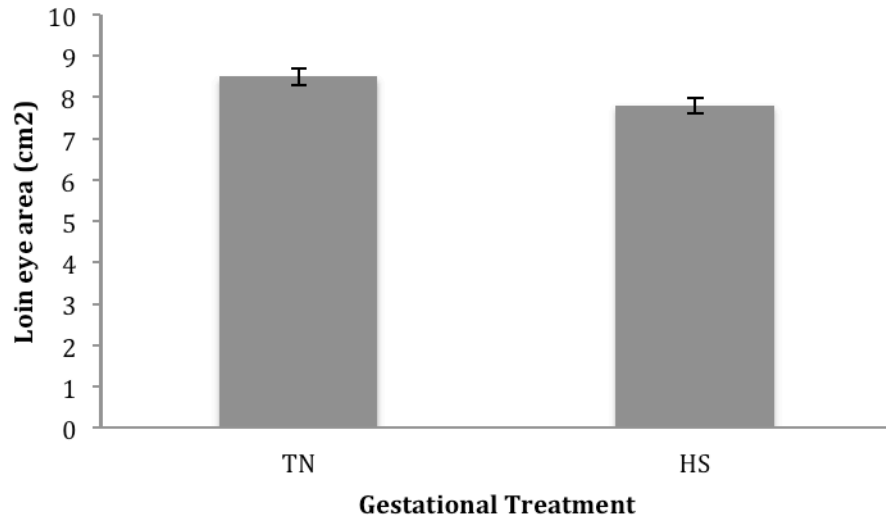


Figure A.4 Loin eye area (LEA) of TN and HS gestational groups. TN gestational group has a greater LEA than the HS gestational group ( $p = 0.0125$ ; adapted from Johnson et al., 2012).

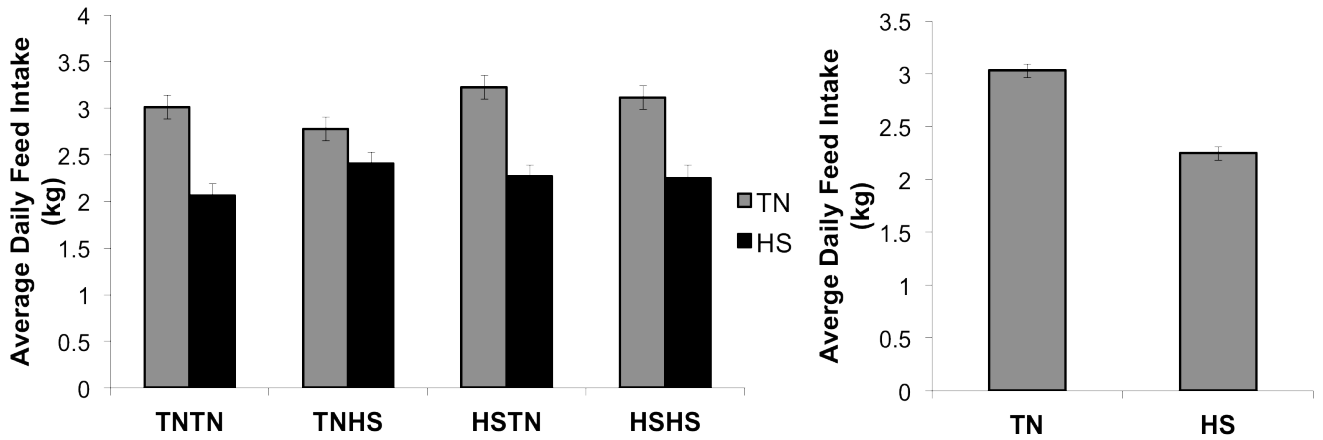


Figure A.5. Average daily feed intake was reduced by postnatal HS (Boddicker et al., 2012). (Left) There was a tendency for an interaction ( $P=0.08$ ) between prenatal and postnatal treatments whereby the degree of HS-induced reduction in feed intake differed between prenatal treatment. (Right) Postnatal feed intake was significantly reduced by postnatal HS ( $P<0.0001$ ).

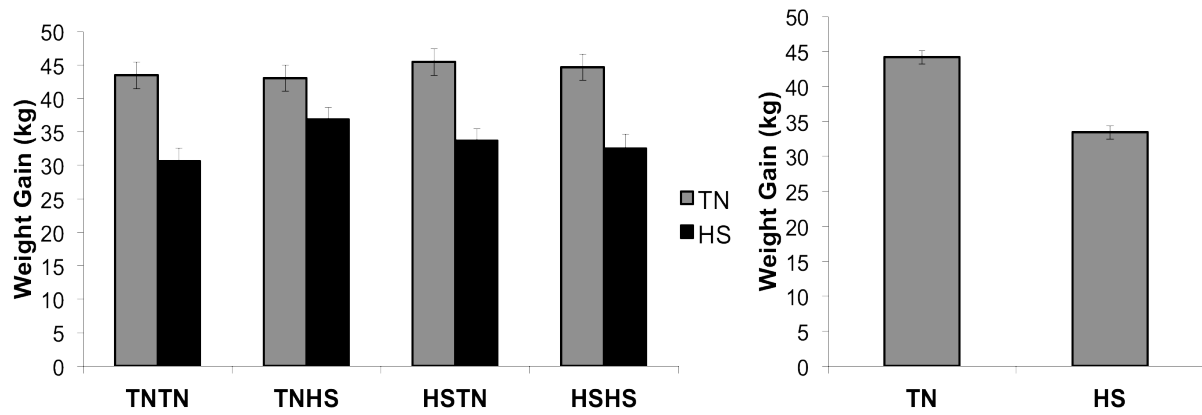


Figure A.6. Weight gain over the postnatal treatment period was affected by postnatal but not prenatal thermal treatment (Boddicker et al., 2012). (Left) There was no significant prenatal treatment effect ( $P=0.46$ ) or interaction between prenatal and postnatal treatments ( $P=0.3$ ). (Right) Weight gain was significantly reduced by postnatal HS regardless of prenatal treatment ( $P<0.0001$ ).

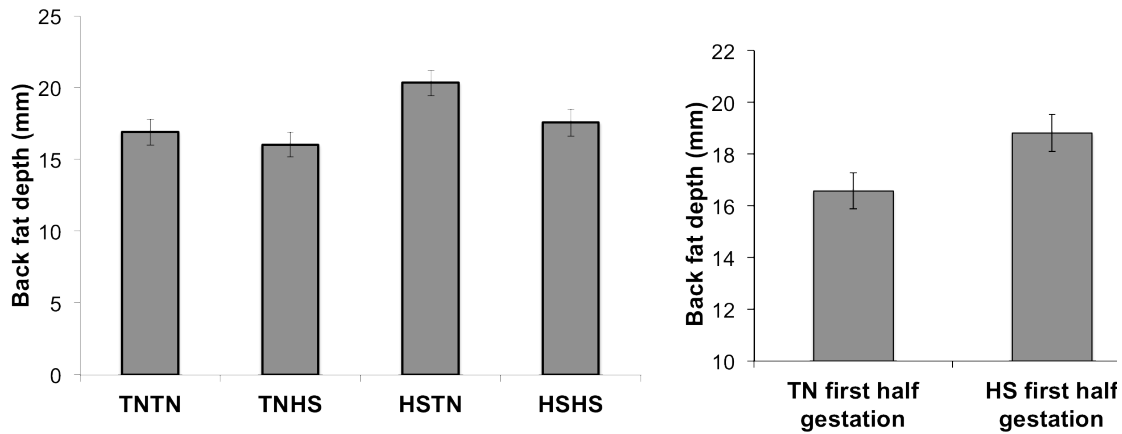


Figure A.7. Postnatal back fat depth was significantly elevated in pigs that experienced prenatal heat stressed during the first half of gestation (Boddicker et al., 2012). There was no significant interaction between prenatal and postnatal treatments with respect to back fat depth ( $P=0.79$ ). (Left) At the end of the postnatal heat stress period, back fat depth was significantly elevated in the HSTN treatment ( $P=0.01$ ). (Right) Contrast between pigs under TN vs. HS conditions for the first half of gestation ( $P=0.03$ ).

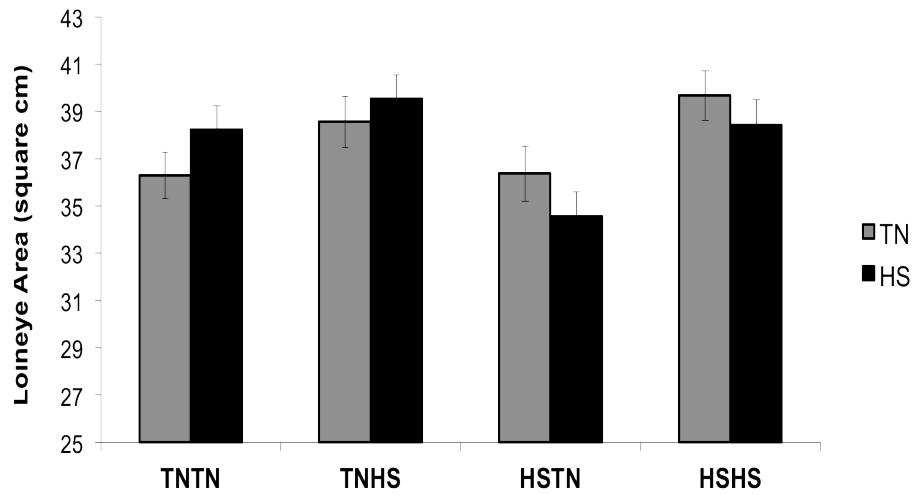


Figure A.8. Prenatal and postnatal thermal treatments interact to regulate loin eye area (Boddicker et al., 2012). There was an interaction ( $P = 0.04$ ) between prenatal and postnatal environments for loin eye area. Offspring from HSHS and HSTN sows had decreased loin eye area after postnatal HS compared to the postnatal TN offspring, whereas offspring from sows in TNTN and TNHS conditions had increased loin eye area following postnatal HS compared to postnatal TN.

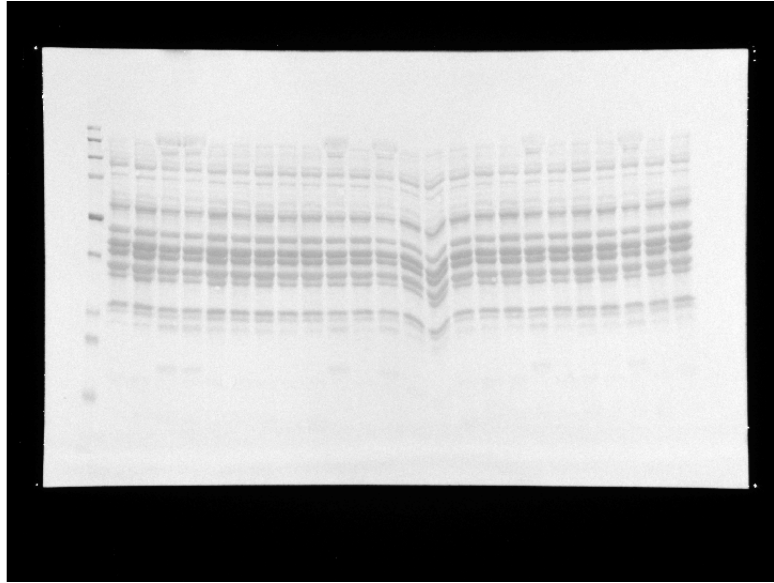


Figure A.9. Ponceau S stain representing equivalence of loading for mixed chronic protein samples 32-153.

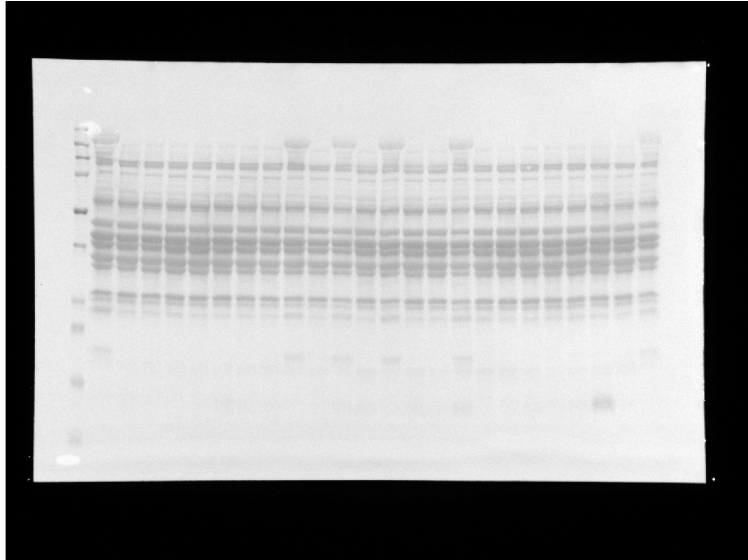


Figure A.10. Ponceau S stain representing equivalence of loading for mixed chronic protein samples 2-174.

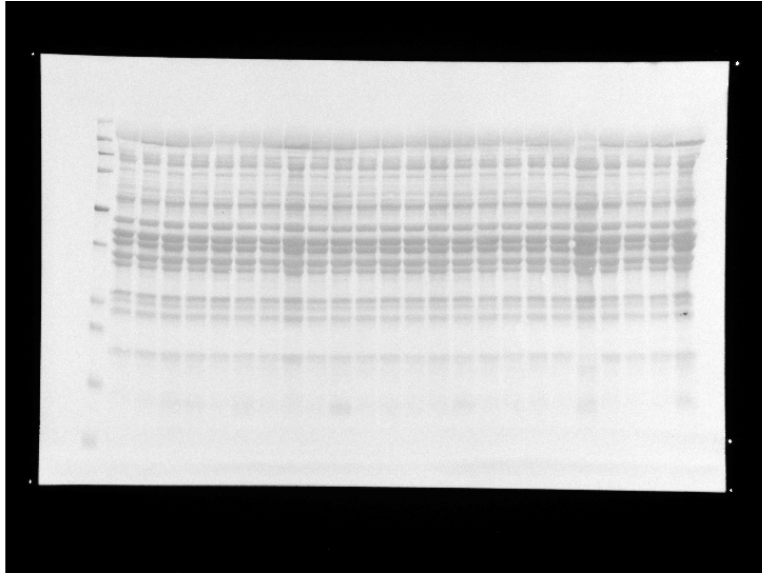


Figure A.11. Ponceau S stain representing equivalence of loading for mixed acute protein samples 7-163.

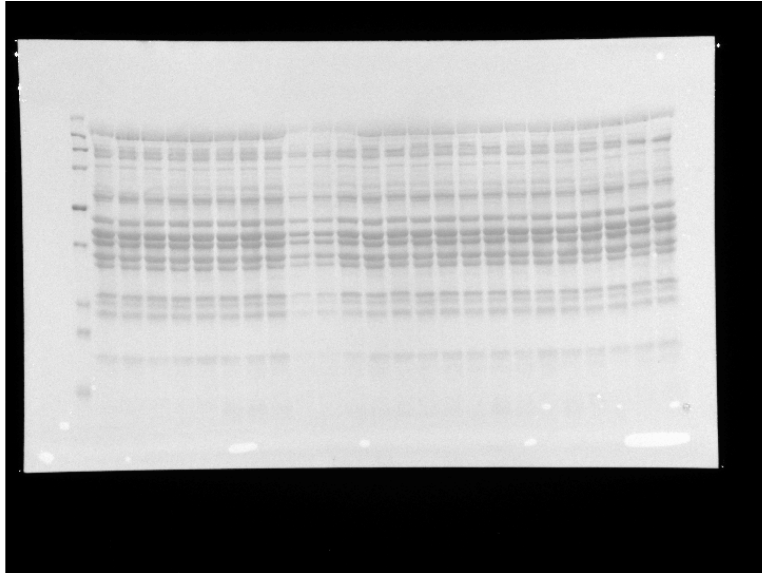


Figure A.12. Ponceau S stain representing equivalence of loading for mixed acute protein samples 23-181.

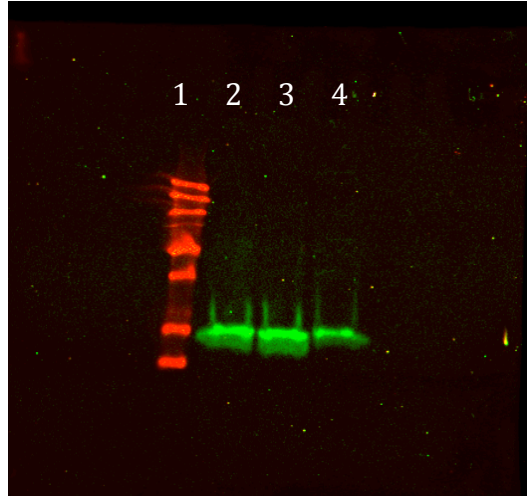


Figure A.13. Validation of 4eBP1 antibody affinity for 4eBP1 in porcine and murine skeletal muscle tissue. Lane 1 was loaded with ladder, Lane 2 was loaded with a chronic heat stress sample. 4eBP1 antibody test detected a dominant band at 15 kd. Lane 3 was loaded with an acute heat stress sample. 4eBP1 antibody test detected a dominant band at 15 kd. Lane 4 was loaded with a mouse skeletal muscle sample. 4eBP1 antibody test detected a dominant band at 15 kd.

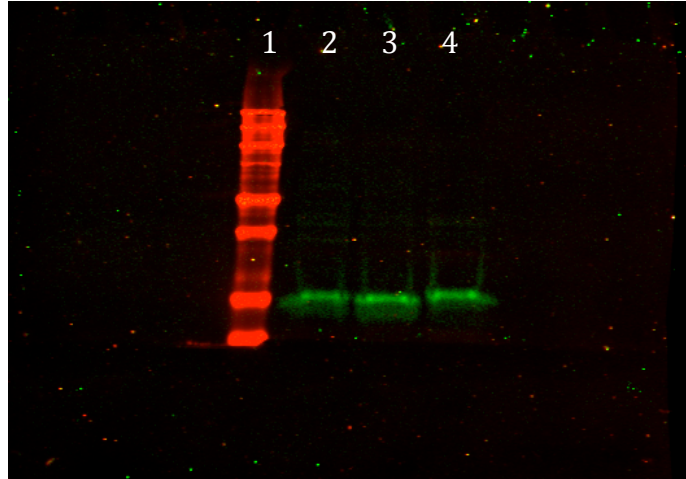


Figure A.14. Validation of phosphorylated 4eBP1 antibody affinity for p-4eBP1 in porcine and murine skeletal muscle tissue. Lane 1 was loaded with ladder, Lane 2 was loaded with a chronic heat stress sample. p-4eBP1 antibody test detected a dominant band at 15 kd. Lane 3 was loaded with an acute heat stress sample. p-4eBP1 antibody test detected a dominant band at 15 kd. Lane 4 was loaded with a mouse skeletal muscle sample. p-4eBP1 antibody test detected a dominant band at 15 kd.

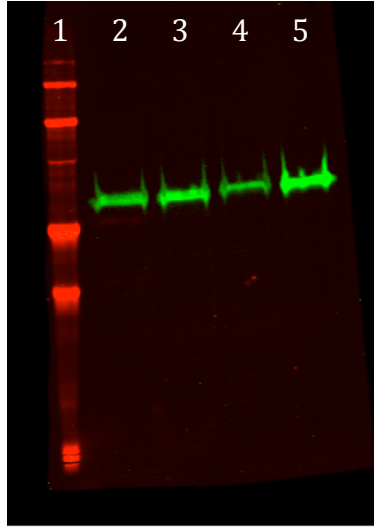


Figure A.15. Validation of Akt antibody affinity for Akt in porcine and murine skeletal muscle tissue. Lane 1 was loaded with ladder, Lane 2 was loaded with a chronic heat stress sample. Akt antibody test detected a dominant band at 60 kd. Lane 3 was loaded with an acute heat stress sample. Akt antibody test detected a dominant band at 60 kd. Lane 4 was loaded with a mouse skeletal muscle sample. Akt antibody test detected a dominant band at 60 kd. Lane 5 was loaded with a mouse liver tissue sample. Akt antibody test detected a dominant band at 60 kd.

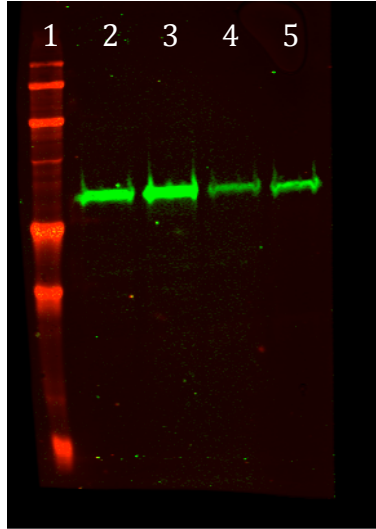


Figure A.16. Validation of phosphorylated Akt antibody affinity for p-Akt in porcine and murine skeletal muscle tissue. Lane 1 was loaded with ladder, Lane 2 was loaded with a chronic heat stress sample. p-Akt antibody test detected a dominant band at 60 kd. Lane 3 was loaded with an acute heat stress sample. p-Akt antibody test detected a dominant band at 60 kd. Lane 4 was loaded with a mouse skeletal muscle sample. p-Akt antibody test detected a dominant band at 60 kd. Lane 5 was loaded with a mouse liver tissue sample. p-Akt antibody test detected a dominant band at 60 kd.

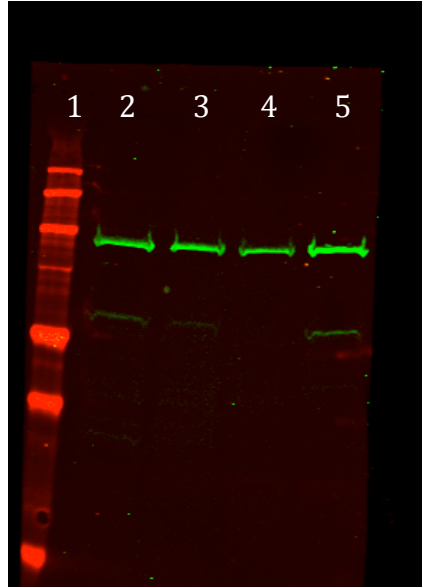


Figure A.17. Validation of EEF2 antibody affinity for EEF2 in porcine and murine skeletal muscle tissue. Lane 1 was loaded with ladder, Lane 2 was loaded with a chronic heat stress sample. EEF2 antibody test detected a dominant band at 95 kd. Lane 3 was loaded with an acute heat stress sample. EEF2 antibody test detected a dominant band at 95 kd. Lane 4 was loaded with a mouse skeletal muscle sample. EEF2 antibody test detected a dominant band at 95 kd. Lane 5 was loaded with a mouse liver tissue sample. EEF2 antibody test detected a dominant band at 95 kd.

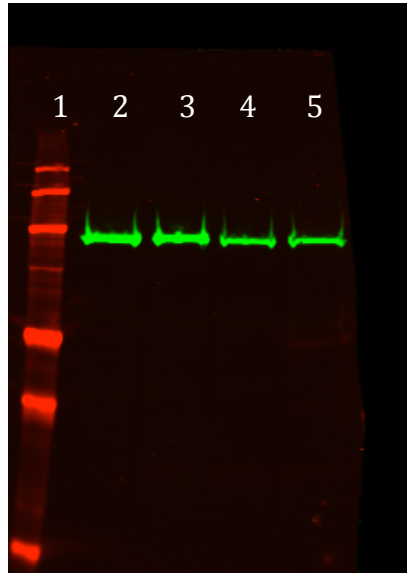


Figure A.18. Validation of phosphorylated EEF2 antibody affinity for p-EEF2 in porcine and murine skeletal muscle tissue. Lane 1 was loaded with ladder, Lane 2 was loaded with a chronic heat stress sample. p-EEF2 antibody test detected a dominant band at 95 kd. Lane 3 was loaded with an acute heat stress sample. p-EEF2 antibody test detected a dominant band at 95 kd. Lane 4 was loaded with a mouse skeletal muscle sample. p-EEF2 antibody test detected a dominant band at 95 kd. Lane 5 was loaded with a mouse liver tissue sample. p-EEF2 antibody test detected a dominant band at 95 kd.

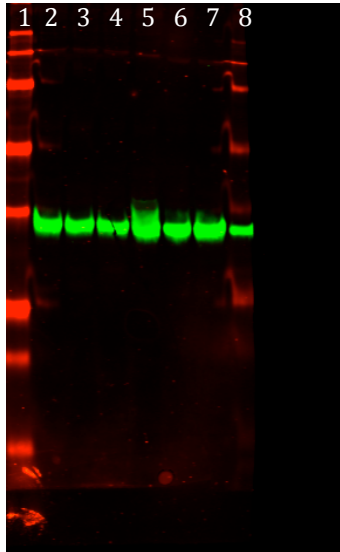


Figure A.19. Validation of GAPDH antibody affinity for GAPDH in porcine and murine skeletal muscle tissue. Lane 1 was loaded with ladder, Lanes 2 -4 were loaded with chronic heat stress samples. GAPDH antibody test detected dominant bands at 37 kd. Lanes 5-7 were loaded with acute heat stress samples. GAPDH antibody test detected dominant bands at 37 kd. Lane 8 was loaded with a mouse skeletal muscle sample. GAPDH antibody test detected a dominant band at 37 kd.

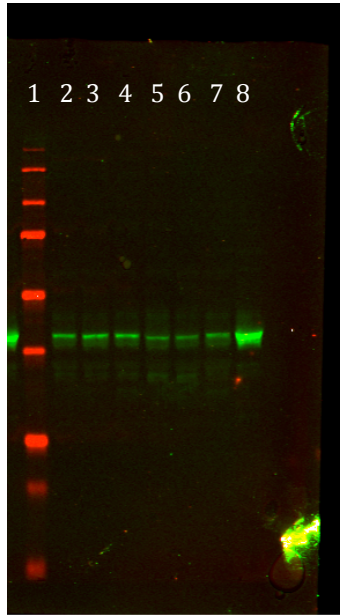


Figure A.20. Validation of PDH antibody affinity for PDH in porcine and murine skeletal muscle tissue. Lane 1 was loaded with ladder, Lanes 2 -4 were loaded with chronic heat stress samples. PDH antibody test detected dominant bands at 43 kd. Lanes 5-7 were loaded with acute heat stress samples. PDH antibody test detected dominant bands at 43 kd. Lane 8 was loaded with a mouse skeletal muscle sample. PDH antibody test detected a dominant band at 43 kd.

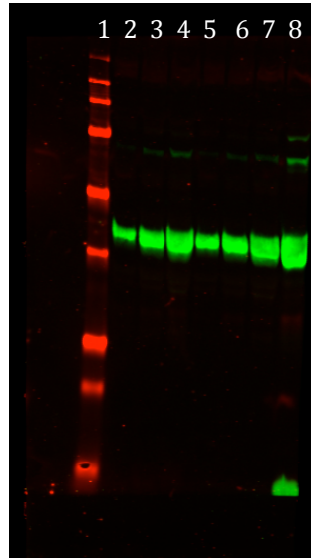


Figure A.21. Validation of phosphorylated PDH antibody affinity for p-PDH in porcine and murine skeletal muscle tissue. Lane 1 was loaded with ladder, Lanes 2 -4 were loaded with chronic heat stress samples. p-PDH antibody test detected dominant bands at 43 kd. Lanes 5-7 were loaded with acute heat stress samples. p-PDH antibody test detected dominant bands at 43 kd. Lane 8 was loaded with a mouse skeletal muscle sample. p-PDH antibody test detected a dominant band at 43 kd.

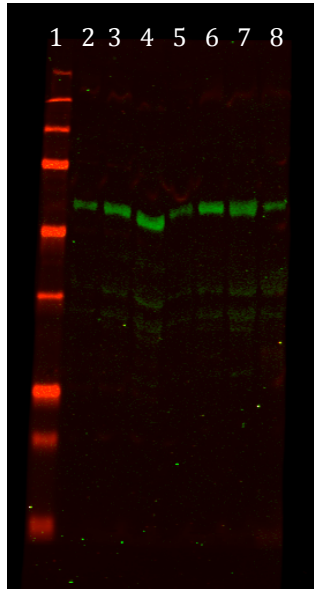


Figure A.22. Validation of PKM2 antibody affinity for PKM2 in porcine and murine skeletal muscle tissue. Lane 1 was loaded with ladder, Lanes 2 -4 were loaded with chronic heat stress samples. PKM2 antibody test detected dominant bands at 60 kd. Lanes 5-7 were loaded with acute heat stress samples. PKM2 antibody test detected dominant bands at 60 kd. Lane 8 was loaded with a mouse skeletal muscle sample. PKM2 antibody test detected a dominant band at 60 kd.

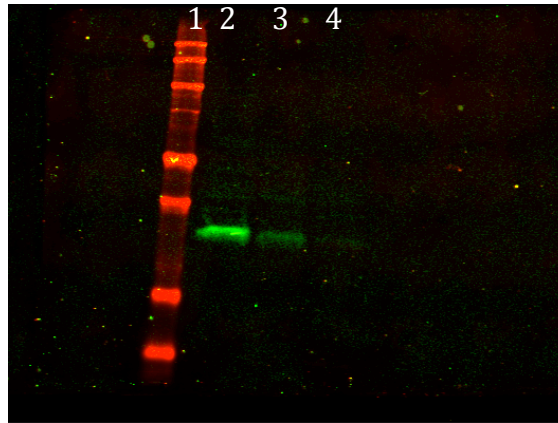


Figure A.23. Validation of phosphorylated Rs6 antibody affinity for Rs6 in porcine and murine skeletal muscle tissue. Lane 1 was loaded with ladder, Lane 2 was loaded with a chronic heat stress sample. p-Rs6 antibody test detected a dominant band at 32 kd. Lane 3 was loaded with an acute heat stress sample. p-Rs6 antibody test detected a dominant band at 32 kd. Lane 4 was loaded with a mouse skeletal muscle sample. p-Rs6 antibody test detected a dominant band at 32 kd.