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Mechanism of adipose tissue specific response to heat stress in pigs.

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MECHANISM OF ADIPOSE TISSUE SPECIFIC RESPONSE TO HEAT STRESS IN PIGS

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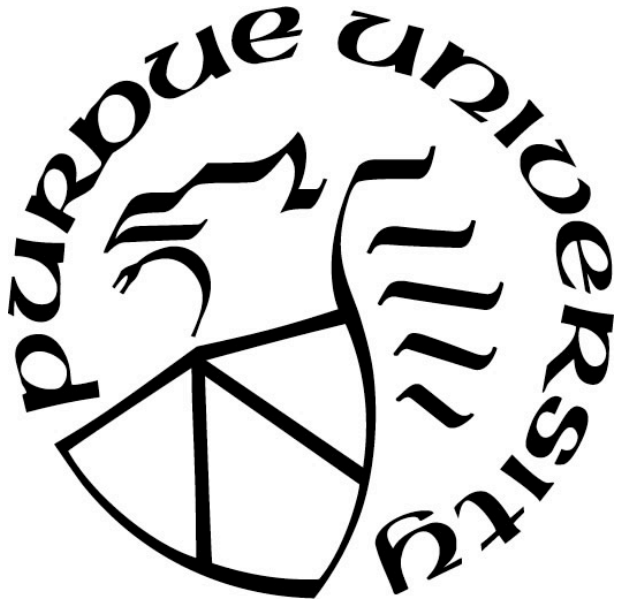

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For my family

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ABSTRACT

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Heat stress (HS) is a major problem facing the swine industry all over the world, especially during the hot summer months. The two major reasons pigs are highly susceptible to elevated ambient temperature are that they have substantial subcutaneous fat layer that serves as a barrier against effective heat dissipation and their lack of functional sweat glands on their skin surface for evaporative heat loss. Paradoxically, HS results in increased fat deposition in pigs. However, the fundamental causes of the increased adiposity during HS are unknown. Therefore, we conducted several *in vivo* and *in vitro* experiments to elucidate the precise mechanisms of HS response in pig adipose tissue. In the first study, we used an in vitro adipocyte differentiation model to characterize cellular responses that occur during differentiation of pig adipocytes in HS. We found the existence of cell autonomous response to HS in pig adipocytes that results in increased adipocyte lipid storage. In the second study, we wanted to elucidate the mechanisms of HS management and their relationship to adipose tissue metabolism. Thirty cross-bred (Ossabaw × Duroc × Landrace) pigs (boars n=15, gilts n=15) were assigned to three treatments for 1 week: 1) control and libitum fed (CON) with environment temperature 20 °C ± 1 °C and ad libitum access to feed, 2) pair-fed (PF) with environment temperature 20 °C ± 1 °C and fed the amount same as HS pigs, 3) HS with environment temperature 35 °C ± 1 °C and ad libitum access to feed. Heat stress led to increased glyceroneogenesis accompanied by induction of phosphoenolpyruvate carboxykinase (PCK1) in adipose tissue without alteration of serum glucose, insulin, FFA and triglyceride concentrations and this revealed that glyceroneogenesis, perhaps through PCK1, plays an important role in increased adipose lipid storage under HS. In the third experiment, we used a commercial-type pig genotype, a Duroc × Yorkshire × Landrace terminal cross, to evaluate effects of both acute (24 h) and chronic (7 d) HS on gene expression responses in adipose and other peripheral tissues. The HS induced a robust adipose tissue response in favor of increased lipid storage. This indicates that adipose tissue might play an

important role in HS adaptation, irrespective of the genotype of the pig. In the fourth study, the effect of PCK1 inhibition with 3 mercaptopicolinic acid (3MPA) on lipid storage and adipocyte response during HS was investigated. Inhibition of PCK1 during HS, adipocytes were less able to induce adaptive responses such as upregulation of HSP70 and triglycerides, and this exacerbated endoplasmic reticulum (ER) stress during HS. Therefore, PCK1 may function to alleviate ER stress that occurs during HS. In the fifth study, we wanted to determine global changes in adipose tissue and adipocyte metabolites and lipids species to see if they could serve as useful biomarkers that could be associated with HS adaptation response. Using metabolomics, lipidomics, and fatty acid profile analysis with gas chromatography, we found that HS induced distinct metabolite profiles. This may have implications for the explanation of the regulation of lipogenesis, fatty acid oxidation and membrane fluidity in adipose tissue during HS. Overall, these studies indicate a potential major role for PCK1 in the increased lipid storage during HS and in the suppression of ER stress that may occur in adipose tissue during HS. Thus, the increased lipid storage in adipose tissue during HS might be a protective mechanism against excessive ER stress and adipocyte death in this tissue.

CHAPTER 1. LITERATURE REVIEW

1.1 Heat stress and its impact on animal production

1.1.1 Global warming as a major climatic trend

Global warming is a major climatic trend, although there is no general agreement regarding its cause or extent (Bernabucci et al., 2010). Global atmospheric concentrations of carbon dioxide, methane, nitrous oxide, and certain manufactured greenhouse gases have all contributed to rising temperatures (U.S. EPA, 2010). Global climate change affects global temperatures, weather patterns, and sea level. The Goddard Institute for Space Studies Surface Temperature Analysis (GISTEMP) is an estimate of global surface temperature change. This estimate indicates that temperatures are rising at a rate of 0.2°C per decade, and that this number is going to rise in the future (US EPA, 2010). Experts have pointed that the trend is accelerating. Some of the expected changes include increases in frequency of severe weather conditions, such as stronger storms, higher winds, and a possible increase in hurricane frequency (NRDC, 2010). The National Research Defense Council (2010) indicated that the entire United Some US states experienced moderate drought in 2007, and the southwestern USA experienced extreme drought in that same year. Coexisting with extreme drought, an elevation in other types of severe weather conditions has also been observed in the USA. Moreover, there has been an increase in the number of heat wave days in the US (Hayhoe et al., 2004). Increased days of heat wave increases the possibility of heat related problems in both humans and animal species. Accompanying these changes, the number of unusually cold days has generally decreased in the USA. This information indicates that future climatic trends are most likely to be accompanied by higher temperatures and higher frequencies of more extreme weather. Therefore, it is necessary to better understand of the mechanism of heat stress on animal production to ensure sustained production of animal protein and by-products for human use.

1.1.2. Heat stress and its effects on swine production.

Heat stress is a situation of environmental temperature rises above the point where heat production and heat loss are balanced. Heat stress induces a considerable economic loss in the swine industry worldwide and adversely effects pig's performance and health. Heat stress is a major concern for the swine industry. Billions of dollars are lost annually globally due to the adverse effects of heat stress animal production. Summer-induced decreased production is well-documented in animal agriculture in the USA (St- Pierre et al., 2003). The swine industry experiences significant seasonal productivity and economic losses from heat stress. For example, it is estimated that the swine industry loses about $202 million for growing-finishing swine and $113 million for sows in the USA (St- Pierre et al., 2003). In total, over $300 million is lost each year due to the negative effect of heat stress on animal production (St- Pierre et al., 2003). The economic losses are still huge, although technology advances in cooling management systems, and other heat stress management strategies can attenuate the effects of thermal stress on health, production, and reproduction of animals.

Although animals are kept in varying environmental conditions, they maintain their body temperature (Tb) within a relatively narrow range and maintain their inner core temperature at a constant value for survival and production. Mammals maintain their body temperature within a narrow range by metabolic heat production. The thermoneutral zone (TNZ) is a range of ambient temperature (Ta) in which animals do not need to adjust their metabolic heat generation or dissipation to counter heat loss or gain (Kingma et al., 2012). Many factors influence the TNZ, such as body composition, energy expenditure, age and gender. The bottom and top boundaries of the TNZ are the lower critical temperature (LCT) and the upper critical temperature (UCT). Thermoneutral zone is dynamic and both LCT and UCT are influenced by relative humidity, solar radiation, air flow velocity, hair coat thickness, etc. and not a fixed value (Hillman, 2009). Two essential mechanisms are used by livestock animals to counter mounting heat stress. One is sensible heat loss and the other one is evaporative heat loss. Sensible heat loss can be achieved by behavioral adjustments such as seeking shades, switching posture utilizing the principles of conduction, radiation and convection and usually is first engaged because these adjustments are

effective at quickly helping to dissipate heat and the energy requirement is low. These heat loss methods are mostly dependent on the temperature gradient between the animal's surface area and the surrounding air environment (Bakken, 1976). The temperature gradient can be reversed and heat flow is directed into the animal when extreme heat wave day occurs. Four basic forms of heat transfer for the pig are conduction, radiation, convection, and evaporation. Evaporative heat transfer is the last means by which an animal increases cutaneous and respiratory evaporative heat loss. This occurs by mechanism such as panting to maintain normal body temperature. In addition, the driving force of evaporative heat loss is the vapor pressure difference between the surrounding environment and an animal (Gomes de Silva and Campos Maia, 2012). Environment cooling systems used in livestock production are based on applying two principles of either increasing heat loss or reducing heat gain and are beneficial to the animal during heat stress (Renaudeau et al., 2012).

Meat quality is affected during HS in swine (Spencer et al., 2005). Meat quality changes in HS include lower carcass weight, changes in meat color, reduced organ weights, increased lipid oxidation, and changes in distribution of fat depots (Toghyani et al., 2012). There are several adverse effects of heat stress on animal production. Heat stress leads to decreases in feed intake (FI), growth performance and increased mortality. The reduction in feed intake ultimately lowers nutrient availability to the animal and results in body weight (BW) loss. Both FI and BW losses can be significant, even with short-term heat stress. Pigs lost an average of 1 kg BW in 6 days under 33°C environmental temperature when FI was reduced by 300 g (Collin, 2001). A 9-d diurnal heat stress treatment cycle (29.4-40.0°C) induced a 0.4 kg/d dry matter intake (DMI) reduction and decreased average daily gain (ADG) of 0.1 kg/d in growing pigs (O'Brien et al., 2010). In addition, in a long-term study (3 weeks), pigs under heat stress at 32°C environmental temperature had a reduction in FI of 771 g/d (approx. 32% reduction) (Renaudeau et al., 2013). In the study by Song et al. (2010), finishing pigs had decreased ADG of 0.87 kg to 0.58 kg and a 26% reduction in FI during a 28-d cyclical HS exposure (Song et al., 2011). Heat stress for 24 h induced a 50% reduction in FI and BW loss of almost 3 kg in growing pigs (Pearce et al., 2013).

There are species differences in heat loss mechanisms that animals employ. Panting is the major mechanism by which heat is dissipated in pigs and sheep. Panting elevates respiration rate which

increases evaporation and ventilation rate in the oral cavity and esophagus (Hales and Webster, 1967). Evaporation via sweating for cooling the core temperature is the major cooling mechanism for mammals such as horses and humans (Fuller et al., 2000). Panting requires muscle contraction, which in itself produces heat. Therefore, sweating is a more efficient way of heat dissipation compared to panting (Fuller et al., 2000). In mammals, heat management mechanisms regulate heat transfer from the thorax to the abdomen to avoid heat stress. This implies that certain animal species may be more susceptible to heat stress based on the major mechanism of heat dissipation that they employ. In particular, pigs have no functional sweat glands and they poorly dissipate heat through evaporative heat loss from their skin surface. This means pigs depend almost entirely heat dissipation by panting (Fuquay, 1981). This problem is complicated in modern pigs because of their high metabolic rate that has resulted from genetic selection for lean growth (Ayuso et al., 2015). Pigs also possess a substantial layer of subcutaneous adipose tissue, which acts as insulation for heat dissipation (Herpin et al., 1993). In summary, the modern pig generates substantial metabolic heat and poorly dissipates this heat. Thus, the pig may be more susceptible to heat stress compared to other species (Patience et al., 2005).

1.1.2 Effect of heat stress on swine carcass quality

Reductions in body fat have been found in pigs under HS treatment (Brown-Brandl et al., 2000; Renaldo and Le Dividich, 1991). Heat stressed pigs had a 26% reduction in feed intake and lower backfat carcass deposition compared to TN pigs (Nienaber et al., 1996). During HS, backfat deposition decreased, whereas leaf pad fat increased (Le Dividich et al., 1987; Rinaldo and Le Dividich, 1991; Katsumata et al., 1996). In pigs, storing more leaf pad fat instead of backfat helps to dissipate more heat through the skin (Katsumata et al., 1996). Carcass and back fat were both decreased in pigs under heat stress treatment (Le Bellego et al., 2002). Muscle growth is favored at the expense of adipose accretion under normal environment temperature, even when nutrient intake is restricted (van Milgen and Noblet, 2003). However, sows exposed to elevated ambient temperature do not lose as much body weight and body condition as do their relative pair fed TN pigs. Adipose tissue carcasses deposition is elevated during heat stress treatment (Collin et al., 2001). Heat stress reduces FI, decreases nutrients available for muscle

protein synthesis, leading to reduced growth performance of animals (Le Bellego et al., 2002; Kerr et al., 2003; Boddicker et al., 2014). Postnatal HS did not alter water, protein, and ash accretion rates, but minimized lipid deposition rates compared to pigs in thermal neutral (Boddicker et al., 2014). However, HS caused reduction of average daily gain, protein accretion, and fat deposition during the finishing phase (Boddicker et al., 2014). Heat stress appears to affect pigs of different sizes differently. Smaller pigs consumed enough feed to allow for protein deposition although they had feed intake reduction (Van Millgen and Noblet, 2003). However, larger pigs did not consume enough energy to support either fat or protein deposition compared to younger pigs under HS. In young pigs, lipid deposition was decreased by 15%, but protein accretion was not affected in HS pigs compared to TN (Boddicker et al., 2014). Therefore, HS effects are more severe with larger pigs. Although the available evidence indicates that HS results in reduction in muscle accretion, and an increase in abdominal fat deposition. the reasons for the alteration in body composition in pigs in heat stress are not fully understood.

Several studies have reported changes in concentrations of ash, lipid, protein, and water in carcasses from HS pigs compared to TN (Stahly et al., 1979; Brown-Brandl et al., 2000; Kerr et al., 2003). Improved animal performance was found in HS when diets were formulated to minimize amino acid (AA) excesses. Reducing dietary crude protein and supplementing amino acid will decrease total heat production in growing pigs whether they are in HS or not. The carcasses of heat stressed pigs had greater concentrations of water, lower concentrations of protein, and lower concentrations of ash than TN pigs (Kerr et al., 2003). However, Stahly et al. (1979) reported no difference in concentrations of water, protein, or ash in carcasses from heat stressed and TN pigs. Brown-Brandl et al. (2000) reported that pigs reared in 30°C had higher concentrations of water, protein, and ash than pigs under TN environment.

Carcass quality was impacted by several factors, including carcass weight, fat (depots and firmness), and weight of primal cuts (Kerr et al., 2005). Heat stress decreased carcass quality due to changes in meat color, decreased carcass weights, and changes in distribution of fat depots (Dalle Zotte, 2005). Increasing HS tended to reduce carcass weight (Zumbach et al., 2008). Carcass from heat stressed pigs had reduced slice weights, and increased lean percentage (Kerr et

al., 2005). More collagen in belly fat was found in heat stressed pigs than TN pigs (Kerr et al., 2005). Collectively, carcass quality is negatively affected by HS.

1.1.3 Heat stress management

Although nutritional strategies are promising and efficient for alleviating the side effects of HS, management and employing heat abatement strategies are still the main approach to relieve HS (Baumgard and Rhoads, 2013). Shade, ventilation, and evaporative cooling can be provided to reduce the side effects of HS. Evaporative cooling system with water in the form of fog, mist or sprinkling with natural or forced air movement, and possibly cooling ponds. Any kind of stressors such as vaccinations and restraint should be avoided during hotter temperatures during the day, as the combination of HS and handling stress together may be too much for animals to manage (Collier et al., 2011). Moreover, social stressors such as regrouping should be avoided during HS as it has been demonstrated to decrease growth performance and feed intake (McGlone et al., 1987). During HS, excessive heat exposure reduces intestinal integrity, and administration of drugs should be avoided as they may exacerbate gastrointestinal integrity disorders and other side effects like cardiovascular side effects (Stoakes et al., 2013). Also, livestock housing design can have a significant impact on the health and well-being of pigs. Room design may affect animal heat production and ventilation to changing nutrition, and environment temperature (Brown-Brandl et al., 2004). Through decreasing body temperature, more feed intake could be encouraged.

Proper nutritional intervention strategies during prolonged HS environment is necessary. Several studies have indicated that heat of nutrient processing (Curtis, 1983), insulin function (Wheelock et al., 2010; Pearce et al., 2013a), and gut integrity (Lambert, 2002; Pearce et al., 2013b) can be significantly affected during HS. It is believed that HS increases basal insulin concentration, implying reduced insulin sensitivity. Proper insulin action is one of the critical factors in adapting to and surviving in heat HS environment. Changes in diet composition or the use of nutritional supplements can be beneficial for HS management. The use of feed additives is an important, flexible and economical method to alleviate side effects of heat stress. Elevating insulin sensitivity may be an effective way to improve animal performance during HS (Rhoads et

al., 2013). Supplementation of diets with chromium (Mertz, 1993), lipoic acid (Diesel et al., 2007), or thiazolidinediones (Ranganathan et al., 2006) are potential means of improving insulin sensitivity (Rhoads et al., 2013).

Adjusting dietary composition is another efficient nutritional strategy to enhance animal health during HS. Alteration in diet composition is necessary during heat stress to maintain nutrient intake, increase dietary nutrient density, or to reestablish homeostasis. Growing pigs will reduce fasting heat production by 18%, daily heat production by 22%, and the thermic effect of feed by 35% under heat stress (Collin et al., 2001). The reduction of HS effect on animal performance is likely due to a reduction in feed intake, and the reduction is beneficial to the animal. Dietary fiber may significantly decrease energy intake. Replacing dietary fiber with energy dense fat sources that can alleviate the side effect of HS on growing animals and increase the amount of energy per kg of feed intake, and this can be a better way to alleviate economic losses due to inadequate nutrient intake under HS (Schoenherr et al., 1989).

Nutritional interventions may be beneficial to the gastrointestinal (GI) tract under HS because the gut is highly susceptible to HS. Heat-stressed pigs redistribute blood flow from their core to the periphery to dissipate heat and less blood flow to the GI tract causing GI tract damage (Hall et al., 1999; Lambert, 2002). Reduced blood and nutrient flow leads to hypoxia at the intestinal epithelium. This compromises intestinal integrity and function. Nutritional strategies such as glutamine (Lima et al., 2005), zinc (Alam et al., 1994; Zhang and Guo, 2009), or betaine (Kettunen et al., 2001; Hassan et al., 2011) may be efficient to reduce side effects of heat stress. Glutamine is a conditionally non-essential amino acid, and also works as the primary energy source for enterocytes (Singleton and Wischmeyer, 2006). Glutamine stimulates intestinal mucosal protein synthesis and protects enterocytes from apoptosis. Glutamine supplementation also has been indicated to benefit intestinal barrier function, and its effect is through activation of heat-shock protein 70 (Lima et al., 2005). Zinc is necessary for normal intestinal barrier function. Zinc deficiency may destroy small intestinal absorptive function. Zinc supplementation can enhance pig's intestinal integrity under both acute and chronic HS (Pearce et al., 2013b; Sanz-Fernandez et al., 2014). Although the mechanism of Zn benefit is poorly understood, the up-regulation of tight junction proteins may play an important role (Zhang and Guo, 2009). Zinc

also benefits the pig as an antioxidant via induction of metallothioneins (Wang et al., 2013). Lastly, betaine is an intracellular osmolyte that also has many labile methyl groups (Cronje, 2007). Betaine may benefit the intestine under intestinal osmotic stress by decreasing the activity of sodium potassium pump (Cronje, 2007). Betaine also leads to reproductive benefits in pigs under HS (Stoakes et al., 2013). Betaine has been shown to relieve the side effects of HS on weight gain, immunity and body temperature in rabbits (Hassan et al., 2011). Despite several reports of the positive effects of betaine in HS treatment, sufficient evidence is still needed to support its mechanism and role in HS alleviation (Stoakes et al., 2013).

Other management strategies can be employed for reducing the side effects of HS. Changes to handling and transportation approaches can be used to decrease economic losses in HS. Transportation is the most stressful event for pigs prior to slaughter. Stress during handling can result in metabolic acidosis that is indicated by an elevation in blood lactate and decreased in blood pH (Ellis, 2003; Peterson et al., 2009). Stress during transportation can result in pig fatigue, injury, poor meat quality and ultimately death (Ellis, 2003; Peterson et al., 2009). Characteristic symptoms of this condition are open-mouthed breathing, cyanosis, and unresponsiveness to stimuli to move. Livestock should be transported at night or early morning in order to minimize the effects of HS during the summer. Proper guidelines should be used with respect to loading density and space requirements of pigs in HS (Grandin 1992).

1.2 Heat stress markers and sensing response

1.2.1 Heat shock proteins and their role in heat stress response

Heat stress results in an instant heat shock response including heat shock protein (HSP) activation. The cellular and molecular responses in livestock are very important as it may lead to identification of biomarker for HS in livestock. Heat shock proteins are a large family of stress proteins that are highly conserved across species. Heat shock proteins are named based on their relative molecular weight. The HSP have chaperon activity, working in the folding, unfolding and refolding of stress-denatured proteins. These proteins can be induced by HS, hypoxia, oxidative stress, and nutritional stress. Their activity is highly induced within several hours of the severe stress and lasts for a few hours or days (Horowitz, 2002). Expression of these HSP is

regulated by activation of transcription factor heat shock factor 1 (HSF1). The activated HSF1 binds to the HSE in the promoter region of HSP genes, resulting in enhanced transcription of HSP mRNA. HSF1 binds to heat shock elements (HSE) and initiates transcription of heat shock genes (Singh and Hasday, 2013).

Most of the protective mechanisms of HSP occur in the cytosol, although HSPs are also found in extracellularly and in the mitochondria (Grubbs et al., 2013) of cells. The expressed intracellular HSP regulate maturation and turnover of intracellular proteins, and this is very important for the maintenance of cellular integrity. HSP were previously thought to be located exclusively in intracellular compartments of the cell. However, researchers showed that HSP70 was also secreted into the blood circulation after tissue necrosis and plays a role in immune response (Basu et al., 2000). The inducible HSP are synthesized in response to stress, as protecting molecules that protect tissues from further injuries. Other HSP such as HSPA5, HSPA9, HSP60 and HSP90 have also been found in extracellular spaces (De Maio and Vazquez, 2013). Release of HSP from the cell may be a result of necrosis, or by a secondary type of unconventional secretory mechanism, active transport mechanism, or lysosome-endosome pathway (Nickel and Seedorf, 2008). But the mechanism by which HSP are exported is still unclear and needs more investigation. Some clinical conditions such as cancer, aging, diabetes, trauma, and infection lead to increase in HSP70 in circulation (Baker et al., 2012; Njemini et al., 2011).

Heat shock proteins protect cells from damage during hyperthermia. Family members of this protein are extensively and conservatively expressed across all species and are present at low levels under normal conditions but transiently increase on cellular stress (Li, 1983; Cairo et al., 1985; Ryan et al., 1991; Moseley, 1997). In many mammalian cells, stress response involves induction of 4 major members of HSP families including 90, 70, 60, and 27 (Arya et al., 2007). Different HSP have separate cellular locations and functions (Feige and Polla, 1994). HSP functions include processing of denatured proteins, management of protein fragments, maintenance of structural proteins, and chaperoning of proteins across cell membranes. Traditionally known as molecular chaperones, HSP binds to unfolded or misfolded proteins and help restore their native conformation (Buchner, 1996; Calderwood et al., 1996; Bouchama and Knochel, 2002). HSP70 plays an importance role in cryoprotection, in nascent folding of

aggregated polypeptide, hence providing stability (Volloch and Rits, 1999). Therefore, HSP70 is extensively used as a biomarker of cellular stress. Expression of HSP70 is generally considered a good indicator under cycled heat stress (Mizzen and Welch, 1988). If the damage from heat exposure is not or cannot be protected by HSP, such as that caused by mitotic catastrophe, programmed cell death through heat-induced apoptosis will happen (Roti Roti, 2008). However, the instant elevation in HSP70 mRNA abundance during acute heat stress treatment is always associated with changing the expression of many enzymes associated with energy metabolism in a skeletal muscle. This indicates that oxidative muscle fibers may be more susceptible to heat stress than glycolytic muscle fibers (Sanders et al., 2009).

Additionally, both heat stress and exercise induced body temperatures increase can increase HSP mRNA or protein level. HSP70 kDa is one of the major HSP, working in protein folding, ubiquitination, renaturation and providing protection during cellular stress (Petrof et al., 2004). Heat shock protein 70 is very sensitive to HS and mainly responsible for mammary cell protection from HS. Heat stress before exercise significantly increases skeletal muscle fiber HSP70 mRNA level in rats (Silver et al., 2012). An increased amount of HSP70 protects skeletal muscles from certain stressors. HSP protein is incredibly sensitive to both exogenous and internal heat production in unacclimated individuals (Magalhães et al., 2010). The effects of environmental heat load and exertional HS on the HSP response are additive. Heat stress and exercise adaptation enhances intracellular levels of HSP70 and inhibits exercise-induced increase in intracellular and plasma HSP70. Exercise-induced HSP mRNA and protein level is not significant because heat adaptation blunts it. For instance, basal circulating HSP70 protein in leukocytes was induced by heat adaptation but not by exercise in heat adapted individuals (Magalhães et al., 2010). Because exercising in the HS environment can further increase core body temperatures. It is still unclear why the HSP response to exercise is blunted in heat adapted individuals. The mechanism of HSP protection during, and after exercise, under HS may provide indications on how heat stress adaptation confers protection to heat stressed farm animals. Heat shock protein 70 also has been proven to regulate pathogen recognition protein such as toll-like receptor (TLR)-4 signaling in the intestine by targeting TLR-4 in immune response (Afrazi et al., 2012). The protein is induced for protecting the mucosal barrier under normal physiologic, pathophysiological and stressful conditions in the intestine (Petrof et al., 2004).

Heat stress results in significant membrane organization alterations. Some members of HSP are actively involved in the restoration and repair of damaged membranes in heat-stressed cells by association at the membranes, although this does not bring any change membrane lipid synthesis and modification (Storey et al., 2002). The lipid components of the cell membranes play a critical role in the cellular responses that happen during acute HS and pathological states. There is a direct relationship between membrane fluidization of membrane lipids and the HSP response. Temporary imbedding of HSP within cell membranes changes membrane fluidity, bilayer stability and membrane permeability, and protects membrane normal functionality during heat stress treatment (Storey et al., 2002). Therefore, HSP are involved directly in the biophysical recovery of cell membranes to maintain the normal membrane state before HS treatment, and this induces inactivation of the membrane-perturbing signal, and then minimizes the mRNA level of HSP genes in a highly responsive feedback manner (Storey et al., 2002). Thus, HS responses may start at the cell plasma membrane.

1.2.2 Mechanism of cellular sensing of heat stress

The physical state of cell membrane is known to be a very sensitive indicator of the most diverse environmental changes. Temperature sensing mechanism is associated with the composition and physical state of cellular membranes. This feature indicates that the cell membrane is an ideal location for primary HS sensing. It is well known that membranes are the main targets of temperature adaptation (Hazel, 1995). Alteration in membrane macromolecular structures is thermally sensitive. Catalytic lipid hydrogenation decreases the degree of unsaturation of fatty acids in the plasma membrane of the cyanobacterium Synechocystis PCC 6803 (Vígh and Joó, 1983; Joó et al., 1991). Low temperature stress *in vivo* induces the mRNA level of an acyl–lipid desaturase (Los et al., 1993). Changes in the environment from a high to a low temperature and its effect on membrane lipids will lead to suboptimal fluidity, causing subnormal membrane function. Cells recognize changes in membrane fluidity and regulate the mRNA level of several genes in HS. The changes in membrane fluidity clearly plays a critical role in the perception of temperature change, and inducing transcriptional induction of desaturases and a reset of heat shock gene transcription (Los et al., 1993). Therefore, modification of membrane lipid

saturation fluidity leads to resetting of the optimal temperature of heat shock response in the yeast (Carratù et al., 1996).

Many cellular components act as sensors to generate the stress signal in HS. The accumulation of denatured proteins under HS induces the activation of the stress protective response (Parsell and Lindquist, 1993; Roussou et al., 2000). Contrary to that, most warm-acclimated organisms that constitute more than 95% of all living species on Earth do not induce HSP when their physiological temperature increases during seasonal changes. Moreover, it is well known that HSP can be induced by a variety of human degenerative diseases and this is associated with the accumulation of denatured proteins. Thus, protein denaturation is a major mechanism for the activation of the HS response and HSP induction.

Heat stress response pathways depend on membrane lipid fatty acid composition in the yeast cells (Chatterjee et al., 1997). Moreover, membrane-linked thermostats could sense HS. The activation of membrane-linked thermostats results from, not only of the increased temperature, but also of the changes in specific membrane lipid composition and physical state. Therefore, cell membranes are greatly dynamic and complex structures that could be involved in HS sensing.

1.2.3 Gender differences in heat stress response

Generally, it is believed that body surface area to mass ratio and body composition (muscle to fat mass) affect heat loss efficiency. It is indicated that a smaller muscle mass to fat ratio are at a disadvantage in heat stress, because it is associated with lower heat loss. In addition, smaller blood volume, coupled with a larger surface for sweating area-to-mass ratio leads to a greater effect of dehydration (Kenney, 1985). Gilts generally have lower muscle mass to fat ratio than boars. At typical slaughter weight for pigs, the intact male (boar) yields a carcass with the least fat and most lean, followed by the female (gilt). This indicates that gilts may be more vulnerable to heat stress than boars.

There are significant differences in growth performance and carcass composition due to gender, and it affects marketing strategy in swine production. Barrows consume more feed and gain BW

more rapidly than do gilts (Ekstrom, 1991). Gilts are more efficient in converting feed to BW gain and deposit a higher percentage of muscle and a lower percentage of fat tissue in their carcasses than do barrows (Ekstrom, 1991). In another experiment, effects of sex, breed and dietary protein level on slaughter weight, performance and carcass traits was studied in crossbred pigs (Christian et al., 1980). Barrows grew significantly faster than gilts. Barrows were reported to have more backfat than gilts.

Cortisol is one of the most common and classical biomarkers to detect stress in pigs. Cortisol is one of the important glucocorticoid in pigs (Bottoms et al., 1972). The upregulation of cortisol is related with hypothalamic pituitary adrenal (HPA) axis activation. Gender is a source of variation of the cortisol level, with concentration in barrows being about 15 % higher than in gilts (Bottoms et al.,1972).

Heat stress leads to increased concentrations of catecholamines and glucocorticoids in many species (Burdick et al., 2011; Fagundes et al., 2008). The effects of HS on immune cells may partially depend on these two hormones. Catecholamines lead to decreased lymphocyte proliferation through decreased IL-1 production (Devaraj et al., 2007). Glucocorticoids regulate T helper (Th) 1 cell to Th2 cell ratio by inhibiting production and responsiveness to IL-12 (DeKruyff et al., 1998; Elenkov et al., 2000). Glucocorticoids suppress cellular immunity and activate humoral immunity by affecting the Th1/Th2 balance. Cortisol induced by HS also suppresses neutrophil phagocytotic function (Salak et al., 1993). In summary, data presented in these aforementioned studies provide evidence of gender differences in response to heat stress due to gender effects on production of stress hormones such as cortisol, and probably catecholamines, and these hormones may mediate gender differences on effect of heat stress on growth performance, carcass composition and heat stress management.

1.2.4 The role of cellular lipids in heat stress response

There are many transporter proteins on the cell membrane. Cell membrane fluidity and stability is affected by heat stress, and this may affect transporter protein function (Hildebrandt et al., 2002). Heat stress activates sphingomyelinase, phosphatase and phosphatidylinositol phosphate

kinase, thus resulting in the phosphatidic acid (PA) and phosphatidyl inositol bisphosphate (PIP2) accumulation (Mishkind et al., 2009). There is increased production of PA under heat stress (Wang X, 2005). Phosphatidic acid affects electrostatic interaction of phospholipids near the head group, thus may affect cell membrane fluidity changes under heat stress.

It has been hypothesized that response to extracellular stimuli depends on the ceramide-enriched platforms which are involved in the propagation of extracellular stress signals across the plasma membrane. The production of HS response second messengers is based on phospholipid-related signaling, especially sphingolipids and cholesterol of membrane rafts. The increase of total cellular ceramide levels is caused by HS which in turn induces membrane stress (Chang et al., 1995). In cell membranes, ceramide is produced at one of the membrane leaflets by sphingomyelin enzymatic cleavage (Grassme et al., 2007). Moreover, increased level of ceramide replaces cholesterol from membrane lipid "cholesterol-raft" and forms "ceramide-raft" (Patra et al., 2008). In fibroblast cells, cholesterol can be transformed to cholesteryl glucoside in heat stress which activates HSF1 and induces HSP70 production (Kunimoto et al., 2000; Kunimoto et al., 2002).

Inositol metabolism has a relationship with intracellular Ca^{2+} pools. The action of PLC induces decrease of PIP2 and PIP levels in HS (Stevenson et al., 1986; Balogh et al., 2010). It has been proved HS could induce PLC leading to the generation of Inositol 1,4,5-trisphosphate (IP3), a common Ca^{2+}-mobilizing second messenger, and diacylglycerol (DAG). Diacylglycerol is a PKC isoforms activator (Bunney et al., 2011). In HS, the inositol phosphate signaling pathways are activated very quickly and their turnover rate is rapid (Stevenson et al., 1986). The increase in Ca^{2}+ during HS may come from instant intracellular Ca^{2+} mobilization from different intracellular origins (Balogh et al., 2005; Kiang et al., 1998). Arachidonic acid (AA) can also be produced from phospholipids by phospholipases C (PLC) and A2 (PLA2), and from DAG by diacylglycerol lipase. Exogenous PLA2 activates AA production, and AA itself activates HS responses (Jurivich et al., 1994; Jurivich et al., 1996).

In yeasts, sphingolipid metabolism is affected by heat HS, indicating that it plays an important role in HS response (Jenkins et al., 1997). Moreover, metabolism of sphingolipids may also be

involved in ceramide formation in HS (Dickson et al., 1997; Cowart et al., 2005). Yeasts with mutation in sphingolipids metabolism are hypersensitive to HS, further indicating the importance of sphingolipids in HS adaptation (Patton et al., 1992; Zanolari et al., 2000). This may be due to the fact that production of sphingoid bases is a HS sensitivity signal to induce cellular responses to HS, including regulation of growth, gene transcription, protein degradation, translation initiation (Cowart et al., 2010).

1.3 Heat stress effects on metabolism

1.3.1 Glucose metabolism

Dietary starch is the major source of glucose for mature swine and other monogastric species (McMillin, 1990). Energy production from glucose in the form of ATP is through glycolysis and pentose phosphate pathway. Glycolysis leads to oxidation of nearly 80-90% glucose in most tissues while the pentose phosphate pathway catalyzes the remaining 10-20% (Wamelink et al., 2008). Glycolysis occurs in the cytoplasm of the cell. During glycolysis one molecule of glucose is converted into two pyruvate molecules and produces 2 ATP. Pyruvate kinase is a rate-limiting enzyme for glycolysis. Glucose is converted into CO_2 and pyruvate, and the pyruvate is then converted to acetyl CoA which enters the citric acid cycle under normal aerobic conditions (Nelson et al., 2008). Intestinal glucose absorption, hepatic gluconeogenesis, as well as glucose uptake and usage by peripheral tissues help to maintained glucose homeostasis (Scheepers et al., 2004). The interplay of various organs and tissues help to maintain glucose homeostasis.

The liver is involved in maintaining glucose homeostasis within the whole body. Glucose is deposited in the liver in the form of glycogen which is then released as glucose via glycogenolysis (Nelson et al., 2008). Hepatic glucose regulation is performed by the glycogen circuit that regulates both the storage of glucose as glycogen as well as glycogen metabolism into glucose-6-phosphate from hepatic glycogen. Glucose metabolism is controlled by the endocrine pancreas through the secretion of insulin and glucagon from the alpha and beta cells. Insulin signals is activated in the “fed” state and decreases glycogen breakdown while activating ATP synthesis (Jones et al., 2012). Insulin secretion is induced by enhanced blood glucose concentrations, gastrointestinal hormones and adrenergic activation. Glucagon is the hormone

secreted from the pancreas and primarily regulates blood glucose levels by stimulating glycogenolysis and hepatic gluconeogenssis (Baile et al., 1983). Glycogen in the muscle and liver is the storage of excess glucose which can be broken down for energy production when it is necessary (Jones et al., 2012).

The change of blood glucose concentrations under HS are unclear because different regulation patterns have been found under heat stress from an increase (Angus et al., 2001), to a decrease (Geraert et al., 1996; O'Brien et al., 2010; Baumgard et al., 2011) or being unchanged (Sano et al., 1999; Rhoads et al., 2009a; Wheelock et al., 2010). These conflicting data might due to differences in experimental settings such as physiological status of animals, species differences, experimental design and heat treatment protocols. Heat stress alters carbohydrate metabolism regardless of the concentration of basal plasma glucose. Exercise-induced dehydration from sweating can raise blood sugar concentration and can lead to heat exhaustion. Exercising in heat elevates blood glucose concentration and this might be due to upregulated hepatic glucose export, coupled with downregulated peripheral glucose uptake in humans (Kozlowski et al., 1985; Angus et al., 2001). Lower insulin secretion after feed consumption as well as muscle degradation are possible mechanisms for maintaining blood glucose concentrations under heat stress. Carbohydrate supplementation is unable to stop the upregulation of hepatic gluconeogenesis under such conditions (Angus et al., 2001). Although insulin dependent glucose uptake by skeletal muscle is downregulated in HS, several studies indicated that glucose utilization is upregulated at the expense of NEFA oxidation (Kozlowski et al., 1985; Angus et al., 2001) in exercising humans. Heat stress acutely activates insulin-independent glucose transport in skeletal muscle (Kozlowski et al., 1985). Moreover, HS affects the respiratory quotient and increases glucose oxidation (Hargreaves et al., 1996). Molecules such as glucose that are more oxidized need less oxygen to be fully metabolized and have higher respiratory quotients.

Whole body HS has been shown to induce an upregulation of glucose metabolism (Dickson and Calderwood, 1979). The increased dependence on glucose as an energy source might explain HS induced glucose absorption enhancement in the intestine (Garriga et al., 2006). Heat stress-induced hepatic glycogenolysis plays a critical role in maintaining blood glucose conetration. The liver is very sensitive to adrenergic signals while adipose tissue lipolysis is not (Baumgard et

al., 2011). In addition to the increase in whole-body glucose consumption, HS leads to significant change in cellular glucose homeostasis. Heat stress leads to elevation of aerobic glycolysis, leading to less dependence on oxidative phosphorylation for ATP production (Baumgard and Rhoads, 2013). Heat stress enhances the ability of muscle to use pyruvate, but not glucose. Heat stress induces elevation of the circulating lactate in growing steers in HS (Elsasser et al., 2009), porcine malignant hyperthermia (Hall et al., 1980). The majority of circulating lactate comes from the metabolism of skeletal muscle (Baumgard and Rhoads 2013).

The pyruvate dehydrogenase complex (PDC) activity is tightly regulated using phosphorylation by pyruvate dehydrogenase kinases, preventing pyruvate's flux through the TCA cycle and oxidative phosphorylation. This directs it towards alanine or lactate synthesis (Nelson et al., 2008). The critical involvement of pyruvate dehydrogenase kinase 4 (PDK4) in regulating glyceroneogenesis through pyruvate sparing in white adipose tissue has been demonstrated by its inhibition or knockdown. Increased mRNA level of PDK4 in heat stressed animals was found compared to TN controls (Furuyama et al., 2003). Therefore, more research is needed towards better understanding of the metabolic regulation of glucose metabolism during HS.

1.3.2 Lipid metabolism

In swine, the primary site for fatty acid synthesis is the adipose tissue, and glucose is the main carbon source for lipogenesis. Glucose is catabolized to acetyl-CoA and then used for de novo fatty acid synthesis. The pentose phosphate pathway provides NADPH for biosynthesis of fatty acids (Nelson et al., 2008). The balance between de novo fatty acid synthesis, fatty acid uptake, esterification, and lipolysis determines the amount of triglycerides deposited in adipose tissue (Chilliard, 1993). De novo fatty-acid synthesis involves some key enzymes. Acetyl Co-A carboxylase, fatty acid synthase (FAS), glucose-6-phosphate dehydrogenase (G-6-PDH), and glycerol-3-phosphate dehydrogenase (G-3-PDH) are the major enzymes that participate in de novo fatty acid synthesis (Guo et al., 2011).

Hormones induced by stress such as catecholamines activate lipolysis and also inhibit insulin. Epinephrine generally activates lipolysis through activiting adipose triglyceride lipase (ATGL)

and hormone sensitive lipase (HSL) and then upregulate non-esterified fatty acids (NEFA) content in circulation. Fat lipolysis and re-esterification cause NEFA release into the circulation (Chilliard, 1993). Pigs have elevated blood NEFA concentration under terminal HS (Marple et al., 1974). Endothelial lipoprotein lipase metabolizes circulating triglycerides in chylomicrons and very-low-density lipoprotein (VLDL) and change them to low density lipoprotein (LDL) and free fatty acids (Guo et al., 2011). Humans have lower fatty acid oxidation under HS compared to TN conditions (Jentjens et al., 2002). Heat stress leads to increase in deposition of adipose tissue in pigs as heat-stressed pigs have a greater amount of adipose tissue compared to those in TN conditions (Collin et al., 2001). This might be due to the metabolic difference between carbohydrates and lipids, although both carbohydrates and lipids act as the main fuels and energy storage compounds in the body.

Hormone sensitive-lipase and ATGL participate in triglyceride hydrolysis. The first fatty acid is hydrolyzed by ATGL to generate diacylyglycerol (DAG). The DAG is then hydrolyzed by HSL to generate monoacylglycerol and one fatty acid (Ahmadian et al., 2007). Monoacylglycerol (MAG) lipase further degrades MAG to a single fatty acid and glycerol. Fatty acids transport is facilitied by fatty acid transport protein (FATP) or fatty acid transporter CD36 (FAT). Fatty acyl CoA synthetases or FATP convert fatty acids into fatty acyl CoA (FACoA). Fatty acid binding proteins (FABP) then bind to and transport FACoA into intracellular compartments. Once inside cellular compartments, fatty acids can regulate transcription through regulation of activities of transcription factors such as peroxisome proliferator-activated receptor α (PPARα), PPARγ and sterol regulatory element binding protein-1 (SREBP-1; Jump et al., 1999; Jump et al., 2005). Intracellular PUFA markedly decreases the mature form of SREBP-1. Also, PUFA appear to prevent SREBP-1 activation by decreasing activites of enzymes that participate in fatty acid synthesis (Jump et al., 1999; Jump et al., 2002). Derivatives of n-3 and n-6 PUFA, have also been shown to be PPAR ligands.

Adipose tissue mobilization is suppressed during HS. Although HS causes a significant decrease in FI, an elevation in carcass lipid deposition has been reported in growing swine (Christon, 1988). Heat stress has been linked with downregulation of plasma NEFA contents in growing pigs (Pearce et al., 2013), chickens (Geraert et al., 1996), rodents (Sanders et al., 2009; Morera et

al., 2012), and exercising humans (Angus et al., 2001). Heat stress decreases lipolytic enzyme activity and upregulates lipogenic enzyme activity in growing pigs (Pearce et al., 2011). In rodents, HS reduces *in vivo* lipolytic rates and *in vitro* lipolytic enzyme activity (Torlińska et al., 1987). Heat stress directly affects fuel selection and overall energetics and this is not mediated by the amount of feed intake. Moreover, mRNA level of adipose tissue LPL is elevated in HS when compared to thermoneutral (TN) ad libitum environments. This indicates that HS induces an upregulation in the capacity to deposit dietary and hepatic-derived triglycerides (Sanders et al., 2009). The downregulation of adipose tissue mobilization downregulation during HS is surprising because HS not only decreases FI, but also increases stress response, with elevated lipolytic signals, such as circulating cortisol and epinephrine (Beede and Collier, 1986). This indicates that heat stress affects lipid metabolism differently from calculated whole body energy balance (Rhoads et al., 2013). Heat stressed animals rely on glucose as energy source more than lipids. Moreover, carcass data shows that pigs (Collin et al., 2001 and Brown-Brandl et al., 2004) have elevated lipid retention under heat stress environments (Yunianto et al., 1997). Plasma non-esterified fatty acids (NEFA) concentration is generally decreased in sheep and cattle exposed to elevated ambient temperature. There is lack of substantial NEFA release in HS, despite marked reductions in feed intake (Sano et al., 1983; Ronchi et al., 1999; Shwartz et al., 2009). As HS causes increase in stress and catabolic hormones (e.g., epinephrine, cortisol, glucagon) (Beede and Collier, 1986), it is a paradox that lipolytic rates is downregulated (Baumgard and Rhoads, 2013).

Increased insulin concentration and sensitivity impacts lipid metabolism in HS, because insulin is an anti-lipolytic and a lipogenic hormone (Vernon, 1992). Heat stress has been linked to increased insulin sensitivity in pigs (Hall et al., 1980). In addition, adipose tissue lipoprotein lipase is upregulated by HS (Sanders et al., 2009), indicating that adipose tissue has an enhanced capacity for uptake and deposition of intestinal and hepatic derived FFA in animals under heat stress (Baumgard and Rhoads, 2013). This suggests that increased capacity for adipose tissue accretion in animals under HS may be due to the restricted capacity for mobilization of adipose tissue lipids and a corresponding elevation in capacity for lipogenesis in this tissue.

1.3.3 Effect of heat stress on protein metabolism

Proteins and amino acids are utilized to synthesize muscle protein, membrane glycoproteins, and are involved in the synthesis of DNA/RNA and enzymes involved in many biochemical metabolite reactions (Young, 1976). The amount of amino acids and ATP available regulates protein synthesis. After transcription initiation, a multistep process is involved in protein biosynthesis. At the ribosome, mRNA has the code for specific polypeptide sequences. Amino acid synthesis leads to formation of a complex protein structure after multiple steps (Nelson et al., 2008). Protein structure is determined by amino acid sequences. The liver metabolites amino acids to incorporate into protein to supply protein for the peripheral tissues (Bergen, 1974). Protein turnover is the balance of protein anabolism and catabolism. Catabolic processes break down complex compounds and molecules to release energy. Protein anabolism indicates the process of protein synthesis from amino acids. During protein catabolism, liver cells break down proteins and use the amino groups to synthesize urea and use the carbon skeletons for synthesis of glucose or fatty acids. Nitrogen excretion can be a marker of protein balance as nitrogen is excreted through urea during protein catabolism (Nelson et al., 2008).

Heat stress affects protein metabolism as it decreases lean tissue accretion in multiple species (Close et al., 1971; Lu et al., 2007). Studies have shown that HS decreases DNA, RNA and protein synthesis (Henle and Leeper 1979; Streffer 1982). Heat stress causes muscle protein breakdown. Tissue protein break down results in an upregulation of blood urea nitrogen (BUN) concentrations. Blood urea nitrogen is often used as a marker of muscle catabolism. Other markers of muscle breakdown include increased plasma Nt-methylhistidine, creatinine, and creatine. Pigs in HS have an upregulation of plasma creatine kinase (CK) which is an indicator of protein breakdown (Aberle et al., 1974) as it metabolites creatine and ATP to phosphocreatine and ADP. Plasma urea nitrogen was induced by HS in pigs (Pearce et al., 2013). Circulating 3-methylhistidine or creatine is the more accurate marker of muscle catabolism. Both of which are induced by HS in pigs (Pearce et al., 2013).

As insulin promotes protein accretion (Allen, 1988), that protein catabolism is increased during HS also indicates that insulin action is also affected during HS. There might be a link between

HS-induced protein catabolism and induction of gluconeogenesis. It has been speculated that protein is broken down in order for the liver to transfer amino acids carbon for gluconeogenesis (Brockman, 1986).

1.3.4 AMP activated protein kinase (AMPK) regulation in heat stress

AMPK is a heterotrimer consisting of a catalytic α subunit and regulatory β- and γ- subunits (Ruderman et al., 2010). In mammals, there are seven genes encoding AMPK: two isoforms of α (α1 and α2), two of β (β1 and β2), three of γ (Y1, γ2 and γ3). For AMPK structure, the α subunit contains a typical serine/threonine kinase domain at N terminus. AMPK is the primary gauge of cellular energy requirements (Hardie, 2008). AMPK is activated when AMP levels increase and energy status is low. AMPK also works to inhibit enzymes participate in ATP-requiring reactions in order to save cellular energy (Kemp et al., 1999).

AMPK may be sensitive to heat stress because heat stress upregulates ATP requirements of the cell while reducing ATP production via oxidative phosphorylation (Corton et al., 1994). AMPK is a potent regulator of skeletal muscle metabolism and it can be upregulated by muscle contraction and is sensitive to a reduction in the ATP/AMP ratio from the utilization of available ATP for muscle contraction (Rasmussen et al., 1998). There is evidence that HS may have different effects in different muscle fiber types and this may be linked to AMPK activity. Activity of AMPK is increased in type 1 skeletal muscle but decreased in type 2 in rodents (Ai et al., 2002). Heat stress has been shown to upregulate hypoxia and oxidative stress (Sato et al., 1991), conditions which are known to upregulate cellular AMPK and HSP70 abundance (Mu et al., 2001; Fryer et al., 2002).

1.3.5 Insulin

Insulin signaling regulates glucose, lipid, and energy homeostasis. Insulin activation increases glucose uptake in adipose tissue and muscle and results in lower blood glucose concentrations (Jones et al., 2012). Insulin signaling induces the glucose transporter GLUT4 translocation to the plasma membrane in muscle and adipose tissue where it regulates glucose uptake (Melmed et al., 2007). The effects of insulin on protein metabolism is independent of glucose or amino acids

transportation into the cell. Insulin affects protein metabolism by increasing the synthesis of proteins in muscle and decreases protein breakdown (Melmed et al., 2007). Insulin regulates how the body uses and stores energy. Insulin regulates glucose storage as glycogen in the liver and also inhibiting gluconeogenesis. Insulin activates lipoprotein lipase in adipose tissue in order to hydrolyze lipoprotein triglycerides and facilitates NEFA entry into the adipocyte. In summary, insulin acts as an anabolic signal to transfer nutrients to muscle and adipose tissue for synthesis (Brockman, 1986).

Although HS reduces FI and increases circulating concentrations of stress hormones, animals in HS do not catabolize adipose tissue. Dependence of heat stressed animals on glucose as an energetic substrate might be due to changes in insulin homeostasis. Relative to pair-fed thermoneutral (PFTN) control animals, HS increases circulating insulin concentration in pigs (Pearce et al., 2013). However, the effects of HS on farm animal whole-body insulin sensitivity remain unknown.

Thermal therapy improves insulin signaling in insulin resistance conditions like obesity (Gupte et al., 2009). The effects of HS on insulin sensitivity are likely mediated by the increase of HSP because similar results are observed by over-expressing HSP72 (Kurucz et al., 2002). Thermal therapy inhibits c-jun amino terminal kinase (JNK) activation which is a stress kinase to inactivate IRS-1 and cause insulin resistance in a HSP-dependent mechanism (Kurucz et al., 2002). It appears that proper insulin action is critical for survival and acclimation to heat stress. Diabetics are more susceptible to heat stress related disease and death (Schuman 1972; Semenza et al., 1999). Insulin administration to diabetic rodents increases survivability in severe HS (Niu et al., 2003). Insulin plays a critical role in HSP regulated protective response and might explain why diabetics with insulin resistance have low HSP72 mRNA level (Li et al., 2006).

1.3.6 Effect of heat stress on tissue metabolism in the liver

The liver is involved in the metabolism of nutrients especially lipid, carbohydrates, protein absorbed from the small intestine, and is important for regulating nutrient homeostasis. The liver synthesizes bile for metabolizing fats and produces important serum proteins such as albumin

and clotting factors. Thus, the liver is important for adaptation to high temperature due to its critical function in maintaining whole body nutrient and energy metabolism. In general, animals decrease feed consumption under HS (Lara et al., 2013), so an important role of the liver will be to maintain concentrations of circulating nutrients such as glucose and triglycerides under HS. Moreover, the liver has antioxidant defense system, including superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST) and glutathione (GSH). Heat stress is always accompanied with oxidative stress (Lin et al., 2006), indicating that the liver may respond to heat stress by elevating the production of biochemical antioxidants (Mahmoud et al., 2003).

The liver plays a critical role in metabolic homeostasis during metabolic stress by regulating blood concentration of metabolites such as carbohydrates, lipids and amino acids. Animals reduce feed intake during HS. This leads to lower dietary availability of nutrients such as glucose. The liver acts as the body's glucose reservoir and can provide glucose to the body either by the breakdown of stored glycogen or through gluconeogenesis. Metabolome and transcriptome data indicate that both glycogenolysis and gluconeogenesis are elevated in the liver under heat stress (Jastrebski et al., 2017).

Beta-oxidation is an energy generating and heat producing process. Break down of fatty acids in the mitochondria produces substrates for the citric acid cycle such as acetyl-CoA and NADH and FADH2 for the electron transport chain. In HS, the concentrations of acyl carnitines concentrations are decreased (Li et al., 2010) Acylcarnitines participate in the transport of fatty acids across the inner mitochondria membrane for beta-oxidation. Thus during HS, fewer substrates are being delivered to the mitochondria for beta-oxidation leading to increased levels of coenzyme A and reduced concentrations of acyl carnitines. The reduction in beta oxidation during HS will minimize elevation of body temperature from fatty acid oxidation.

The liver is a major organ for metabolizing excess carbohydrates and proteins into fatty acids and triglyceride. Fatty acids are mostly stored as triglycerides, and can be broken down to meet different metabolic demands. Under HS, the concentrations of fatty acids are either down-regulated or unchanged, except the increase of myristate, myristoleate, and palmitoleate (Jastrebski et al., 2017). In HS, the concentration of acetyl-CoA carboxylase (ACC), which

metabolites acetyl-CoA to malonyl-CoA is increased (White et al., 2013). Fatty acid synthase (FAS) was detected but unchanged under heat stress treatment (White et al., 2013). The concentration of stearoyl-CoA-9-desaturase (SCD), an enzyme which generates myristoleoyl-CoA and palmitoleoyl-CoA from myristoyl-CoA and palmitoyl-CoA respectively, is induced by heat stress (White et al., 2013).

The concentration of glycerol and glycerol-3-phosphate (G3P) is also increased in heat stress (Jastrebski et al., 2017). These two metabolites are important as precursors for the triacylglycerol backbone. It has also been reported that that glycerol kinase (GK) concentration is reduced during heat stress (Jastrebski et al., 2017). It is speculated that G3P is being synthesized through glyceroneogenesis. Glycerol 3-phosphate is generated during glyceroneogenesis from precursors other than glucose. Thus, G3P may be coming from precursors such as pyruvate, which is elevated in HS (Reshef et al., 2003). In the mammalian liver, it is reported that the rate of fatty acid re-esterification resulting from glyceroneogenesis was higher than the rates for glycerol conversion to glyceride-glycerol in adipocytes (Reshef et al., 2003). Glycerol 3-phosphate is synthesized by reducing dihydroxyacetone phosphate (DHAP). In HS, the enzyme that regulates the conversion of G3P from DHAP is glycerol-3-phosphate dehydrogenase (GDP2). It is speculated that the equilibrium concentrations of substrates drive the reaction towards synthesizing G3P from DHAP, leading to lower concentrations of available DHAP in animals (Jastrebski et al., 2017). Palmitoleic acid is esterified into TAG molecue. And the TAG is then incorporated into VLDL and transported from the liver to adipose tissue for deposition.

1.3.7 Role of phosphoenolpyruvate carboxykinase (PEPCK-C) in glyceroneogenesis

The main function of PEPCK is regulating the following metabolic steps: gluconeogenesis, glyceroneogenesis, the synthesis of serine, and the conversion of the carbon skeletons of amino acids, such as glutamine and glutamate, to P-enolpyruvate (via PEPCK) and then to pyruvate (via pyruvate kinase) for subsequent oxidation in the citric acid cycle as acetyl-CoA. The enzyme catalyzes the reaction of Oxalacetate + GTP ----→ P-enolpyruvate + GDP + CO_2. All eukaryotes have a gene for mitochondrial (PEPCK-M) and cytosolic (PEPCK-C). The genes for human

PEPCK-M and PEPCK-C have the same code: 10 exons and 9 introns (Beale et al., 1985; Modaressi et al., 1998). The size of the introns in PEPCK-M is the main difference.

Gluconeogenesis is the pathway by which PEPCK catalyzes the synthesis of glucose from protein amino acids and other substrates in liver. Gluconeogenesis helps maintain blood glucose concentrations when glucose concentration is low. PEPCK also regulates glyceroneogenesis in adipose tissue. The production of a glycerol from glyceroneogenesis is very important for the synthesis of triglycerides backbone and the release of fatty acids in to the blood stream. PEPCK also could be a cataplerotic enzyme because it functioning in removing citric acid cycle anions for either the biosynthetic steps or in TCA cycle with the subsequent oxidation of these compounds to CO_2 (Yang et al., 2009).

One of the key regulators of free fatty acids to triglycerides ratio is the cellular concentration of glycerol-glyceride to form the backbone for triglycerides. Upregulating the incorporation of FFA into TG may occur by elevating the activity of PEPCK, and this changes the cellular balance of free fatty acids and triglycerides towards a higher concentration of triglycerides. This happens in both the liver and adipose tissue (Reshef et al., 2003).

1.3.8 PEPCK-C regulation

PEPCK gene promoter region contains several response elements: PPAR response elements, insulin response elements, glucocorticoid response elements, thyroid response elements, cAMP response elements. Therefore, transcriptional factors could bind these response elements to upregulate PEPCK mRNA level.

Since PEPCK-C is involved in FA recycling, it would make sense that FA affects mRNA level of this gene. It has been shown that unsaturated FAs, either mono- or poly-unsaturated, are strong and rapid direct inducers of PEPCK-C transcription in 3T3-F442A adipocytes (Antras-Ferry et al., 1994; Antras-Ferry et al., 1995)

PEPCK mRNA level is also negatively mediated by several circulating hormones such as glucagon and glucocorticoids (Hanson et al., 1997). Each of the hormones exerts its effect through hormone response elements in the PEPCK gene promoter sequence. For example, glucocorticoids reduce the transcription rate of the PEPCK-C gene via a mechanism involving the glucocorticoid receptor (Franckhauser et al., 1995).

There is no known post-translational regulation of the enzyme, thus changes in PEPCK-C activity directly reflect modifications of PEPCK-C mRNA synthesis and/or degradation (Forest et al., 1997). The PPARγ2 binding site is required for the adipose tissue-specific expression of PEPCK-C gene (Devine et al., 1999). This not only reduces the cellular concentration of free fatty acids in fat tissue, but it reduces the amount of free fatty acids released in to the blood.

AMPK negatively regulates the transcription of PEPCK gene by phosphorylation of its transcription factors and co-activators (Lochhead et al., 2000). AICAR response element binding protein (AREBP) phosphorylation by AMPK and then repress transcription of the PEPCK gene. AREBP is phosphorylated at Ser 470 by AMPK (Erina et al., 2006). Phosphorylation produces a conformational change in AREBP and that this change results in decreased DNA-binding activity.

There are several lines of evidence suggesting that the AMPK signaling pathways can lead to the inhibition of PEPCK mRNA level. The adipocyte-derived hormone adiponectin, which probably plays an important role in the regulation of glucose and lipid metabolism, represses PEPCK mRNA level through activation of AMPK (Yamauchi et al., 2002). Moreover, the antidiabetic drug metformin might exhibit some of its effects through the activation of AMPK resulting in down-regulation of gluconeogenesis via a decrease in PEPCK mRNA level (Zhou et al., 2001).

In rats fed a high protein, low carbohydrate diet, dietary fatty acids played an important role in promoting glyceroneogenesis in adipose tissue (Brito et al., 2006). Although glyceroneogenesis plays an important role in providing the 3-glycerol phosphate in fatty acids esterification in adipose tissue and the liver, the low mRNA level of PEPCK-C in skeletal muscle suggests that glyceroneogeneisis is minimal in this tissue. Skeletal muscle contains triglyceride stores, a low

level of PEPCK-C activity, and excessive lipid accumulation in skeletal muscle is predominantly responsible for the insulin resistant state observed in Type 2 diabetics (Varady et al., 2007).

Overexpression PEPCK-C gene in adipose tissue in mice leads to adipose tissue hypertrophy (Franckhauser et al., 2002). The conversion of [14C] pyruvate to triglyceride glycerol in adipose tissue was increased 2-fold and 2.5-fold, in heterozygous and homozygous transgenic mice respectively, compared to their relative control mice. Moreoever, reesterification of FFA was nearly 2-fold greater than controls in transgenic mice. The upregulated G-3-P synthesis through the glyceroneogenic pathway results in re-esterification of a higher amount of FFA in transgenic mice expressing the AP2-PEPCK chimeric gene (Franckhauser et al., 2002). This indicates that the dysregulation of glyceroneogenesis may affect lipid accumulation and results in obesity (Franckhauser et al., 2002).

1.4 Endoplasmic reticulum (ER) stress

1.4.1 ER stress and unfolded protein responses signaling

The ER is a very complex and important cellular organelle. The composition of the ER includes a single continuous phospholipid membrane that is comprised of the outer nuclear envelope, flattened peripheral sheets with ribosomes called rough ER and an extensive network of smooth tubules called smooth ER that expand the cell. The ER has many different cellular functions such as protein folding. In the smooth ER, drug detoxification, fatty acid and steroid biosynthesis and Ca^{2+} storage also takes place. In the rough ER, folding and post-translational processing of membrane bound and secreted proteins occurs. The ER compartments also cooperate with the chaperone systems such as glycosidases, Ca^{2+}-dependent chaperones and members of the protein disulphide isomerase (PDI) family.

ER stress occurs during physiological or pathological environments that disrupt the protein folding compartment, and affect the proper function of ER's protein folding machinery (Gotoh et al., 2011). The cellular responses to ER stress includes the activation of the unfolded protein response (UPR) which is a set of signaling pathways, and a catabolic reaction called autophagy, and cell death (Gorman et al., 2012).

1.4.2 ER stress and heat stress

Elevated reactive oxygen species (ROS) production has been found in heat and oxidative stress (Schiaffonati et al., 1990; Salo et al., 1991). An increased flux of ROS was found in intestinal epithelial cells exposed to 45°C for 20 min (Flanagan et al., 1998) and increased superoxide formation was examined in diaphragm muscle of heat-stressed mouse (Zuo et al., 2000). Broiler chickens under acute HS had greater concentration of malondialdehyde (MDA), a marker of lipid peroxidation, in skeletal muscle, than control (Mujahid et al., 2009). Heat stress also led to downregulation of oxidative metabolism in rats, thereby leading to significant lowering of ATP concentration in the cardiomyocytes (Qian et al., 2004) and uncoupling of oxidative phosphorylation causing an increase in ROS production and oxidative stress. In HS, transcript and protein concentrations of ROS scavenging enzymes were elevated (Rainwater et al., 1996).

Aged animals (Hall et al., 2000; Zhang et al., 2003) as well as diabetics (Rolo and Palmeira, 2006) are linked to higher production amount of superoxide in mitochondria. Mitochondria may be more susceptible to HS exposure, and further upregulation of ROS generation and subsequent oxidative damage (Haak et al., 2009).

1.5 Metabolite and lipid analysis of adipose tissue in heat stress

1.5.1 Metabolomic and lipidomic signature

Metabolomics is used to recognize, measure and interpret complex time-related concentration, activity and flux of endogenous metabolites in cells, tissues, and other biosamples. Metabolome examination has been found useful in the context of analysis of metabolites for phenotypic profiling of Escherichia coli cells (Tweeddale et al., 1998). Although there may be negligible whole phenotype change (Cornish-Bowden and Ca´rdenas 2001), metabolomics is an excellent way to analyze sensitivity of the biological system (Goodacre et al., 2004). Therefore, metabolomics is seen as a practical approach to understanding biological systems accompanied by proteomics and transcriptomics.

Biomarker identifications of metabolites have already been proven useful in animals exposed to various stresses from the environment (Soga et al., 2006; Loftus et al., 2008). Moreover, high throughput profiling of metabolic snapshots has been carried out in a variety of rat tissues (Chu et al., 2004). Applying metabolomics for biomarker discovery is a very active research area. Utilizing metabolite profiling to generate quantitative records of metabolites from control populations and test subjects is common. Data analysis is applied to help understand the metabolites results and predicting which are discriminatory for a disease situation and which of these could be used in medicine. Statistically significant difference between individual metabolites between healthy and diseased subjects can be detected by univariate analyses of ANOVA, Student's t-test or non- parametric equivalents (Goodacre et al., 2004).

Liquid chromatography mass spectrometry (LC/MS) is useful for metabolite profiling. LC/MS is also applicable to high throughput applications which has a reasonable dynamic range combined with potential for biomarker identification based on the spectral data (Goodacre et al., 2004). LC/MS is not specific to particular classes of compounds and can be extremely sensitive (Goodacre et al., 2004). Polar molecules could be directly analyzed by MS due to the application of electrospray ionization (ESI) technique.

Metabolite markers are more robust and representative of signaling responses because they represent a convergence of multiple signals to regular metabolic pathways (Goodacre et al., 2004). Finally, due to the small size of metabolites, instrumentation needs for metabolite detection are more established and less expensive than for proteomics analysis.

Use of metabolomics analysis in HS experiments would allow for the quantitative determination of global metabolite changes in HS and identification of biomarkers of heat stress response.

In summary, HS affects numerous physiological processes including nutrient partitioning and lipid metabolism. The fundamental causes of the increased adiposity in pigs exposed to HS are still not fully known, although there is evidence that HS increases activity of lipogenic enzymes such as LPL in adipose tissue of pigs. However, existing literature indicate that adipose-tissue specific responses are an integral part of the overall adaptive response to HS in pigs.

Understanding such responses are critical for obtaining better insight into the overall mechanisms of HS response and adaptation in pigs.

Therefore, this dissertation sought to investigate: a) direct effect of heat exposure on pig adipocyte differentation; b) whether increased lipid storage during heat stress represents a protective mechanism of heat stress adaptation in pigs ; c) the importance of PCK1 activation and lipid storage in the adaptive response to heat stress; d) the use of metabolomics approach to determine the identity of metabolites that were altered in response to heat stress.

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CHAPTER 2. HEAT STRESS ENHANCES ADIPOGENIC DIFFERENTIATION OF SUBCUTANEOUS FAT DEPOT-DERIVED PORCINE STROMOVASCULAR CELLS

2.1 Abstract

Heat stress (HS) results from excessive heat load on animals such that all adaptive mechanisms employed to dissipate the heat do not return the body to normal body temperature. In pigs, HS results in increased fat deposition compared with pair-fed animals in a thermoneutral environment. Although there is evidence that HS increases activity of lipoprotein lipase (LPL) in adipose tissue of heat stressed pigs, the fundamental causes of the increased adiposity are still unknown. It remains unclear whether HS directly alters metabolism in adipocytes. Therefore, to understand the mechanism of HS effects on porcine adipocytes, we used an in vitro adipocyte differentiation model was used to characterize cellular responses that occur during differentiation of pig adipocytes. Preadipocytes (stromovascular cells) were differentiated for 9 d at a normal (37°C) or high temperature (41.5°C) under 5% CO2 for a total of 9 days. Expression of HS genes such as heat shock proteins (HSP: HSP27, HSP60, HSP70, HSP90), adipogenic markers peroxisome proliferator-activated receptor γ (PPARγ), CCAAT box/enhancer binding proteins α (C/EBPα), fatty acid synthase (FAS), adipocyte protein 2 (aP2), fatty acid translocase 36 (CD36), fatty acid transport protein 4 (FATP4), fatty acid transport protein 6 (FATP6), LPL, glucose transporter protein type 4 (GLUT4), phosphoenolpyruvate carboxykinase 1 (PCK1 or PEPCK-C), glycerol kinase (GK) and adipokines (adiponectin and leptin) were determined by real time-PCR, and immunoblotting or ELISA. Cellular triglyceride (TAG) and ATP concentrations were also determined. As expected, HS increased ($P < 0.05$) the expression of HSP genes. There was no heat treatment effect on the level of PPARγ, although C/EBPα was induced ($P < 0.05$) in HS. So, it remains unclear whether HS affects adipocyte differentiation. However, elevated temperature led to increased mRNA levels of genes involved in fatty acid uptake and TAG synthesis (FAS, aP2, CD36, FATP4, FATP6, LPL, GLUT4, PCK1 and GK). This is supported by increased cellular TAG under HS. Therefore, HS promotes increased adipocyte TAG storage, perhaps through upregulation of genes involved in fatty acid uptake and TAG synthesis.

Key words: adipocyte differentiation, heat stress, swine

2.2 Introduction

Heat stress (HS) occurs when animals are unable to dissipate excess body heat (West, 2003). Pigs are especially vulnerable to HS because they lack functional sweat glands on their skin. Pigs exposed to HS suffer reduced performance (Spencer et al., 2005). Effects of HS in pigs include increased plasma insulin and increased N(τ)-methylhistidine, but decreased plasma NEFA (Pearce et al., 2013a). Heat stress results in increased carcass fat (Bridges et al., 1998; Collin et al., 2001) in the pig when compared with pair-fed animals in thermoneutral temperature. Although precise mechanisms of this effect are unknown, elevated plasma insulin, a lipogenic hormone, may be a contributing factor during HS (Pearce et al., 2013a). Increased insulin concentration may also be responsible for the increased expression and activity of lipoprotein lipase (LPL), another adipogenic enzyme, in the adipose tissue of heat stressed pigs (Kouba et al., 2001).

Despite the likely implication of insulin in the increased fat mass of heat stressed pigs, there are significant inconsistencies in the regulation of serum insulin concentrations in pigs under HS (Williams et al., 2013; Pearce et al., 2014), suggesting that additional mechanisms may be involved. Experiments are needed to elucidate the precise mechanisms of HS response in the adipose tissue of the pigs to establish implications for the increased adipose accretion in pigs under HS. Using an in vitro differentiation model of pig preadipocytes exposed to mild HS recapitulates the effects of medium to long-term HS in pigs. Unlike acute HS, chronic HS induces more adaptive responses and non-lethal metabolic alterations (Horowitz, 2002), more akin to an in vivo adaptive response in pigs. Therefore, the objective of this study was to determine the molecular mechanism of chronic HS effect on adipocyte differentiation and lipid storage in subcutaneous fat-derived porcine stromovascular cells (SVCs).

2.3 Materials and Methods

Primary cell isolation and cell culture

All animal care and protocols described in these experiments were approved by the Purdue Animal Care and Use Committee. Isolation and culture of primary porcine preadipocytes were based on the protocol described previously (Ramsay, 2005). Briefly, preadipocytes (SVCs) were isolated from the inner layer of subcutaneous adipose tissue from male juvenile piglets (< 7 d old). Excised tissue was kept immediately in buffered saline (0.15 M NaCl, 10 mM HEPES, pH 7.4) at 37°C. Tissue was thoroughly minced through repeated cutting with a pair of scissors and then incubated with a cocktail (10 mM NaHCO3, 10 mM HEPES, 5 mM D-glucose, 120 mM NaCl, 4.6 mM KCl, 1.25 mM CaCl2, 1.20 mM MgSO4, 1.20 mM KH2PO4, 3% BSA) containing collagenase (Collagenase type I, 1mg/mL, Worthington Biochemical Corp., Lakewood, NJ) in a shaking water bath at 37°C for 45 min at 120 oscillation/min. Digested sample was centrifuged at 2000 × g for 10 min at 4°C to separate the floating adipocytes from the precipitated SVC pellet. The SVC was washed twice in the digestion cocktail. Afterwards, the pellet was suspended in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) medium (Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum (Mediatech, Manassas, VA), 1% antibiotic-antimycotic (Sigma Aldrich, St. Louis, MO) and incubated in a humidified incubator with 5% CO_2 and 95% air. After reaching confluence, cells were differentiated under normal (37°C) or HS (41.5°C) temperature environments for 3, 6 or 9 d in a differentiation medium: DMEM/F12 with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic, 1μM insulin, 1μM dexamethasone (1mM), 1μM rosiglitazone, 1 μM biotin, 1μM triiodothyronine (T3), 1 μM pantothenic acid. Medium was replaced every three d and rosiglitazone was removed from the differentiation media after d 3 of differentiation. Cells showed visible signs of lipid accumulation from d 6 of differentiation and were fully lipid filled by d 9.

Oil red O staining for cellular lipids

Oil red O staining was conducted following previously described protocol (Ramírez-Zacarías et al., 1992) with slight modifications. Briefly, cells in 24-well plates were washed twice with 1×PBS and immediately incubated in 10% formalin for 5 min at room temperature. The formalin was discarded and fresh formalin was added for another hour after which cells were washed with

60% isopropanol and then incubated in Oil red O working solution for 10 min. Cells were finally washed with deionized distilled water and photographed on a Nikon Eclipse TE 2000-U microscope (Nikon, Tokyo, Japan).

Gene expression analysis

Gene expression was measured through real time-PCR. Total RNA from adipocytes was extracted using QIAzol lysis reagent (Qiagen, Valencia, CA), based on the manufacturer's protocol. Extracted RNA was dissolved in nuclease-free water (Ambion, Austin, TX). Concentrations of RNA were determined with a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Integrity of RNA and genomic DNA contamination were checked by electrophoresis on 0.8% agarose. One μg RNA was reverse transcribed with Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, WI). A Bio-Rad MyiQ thermocycler (Bio-Rad, Temecula, CA) was used to conduct the PCR assays. The PCR reaction mixtures contained 0.5 μg of cDNA, 0.075 nmol of forward and reverse primers respectively, and RT2 SYBR Green qPCR master mix (SABiosciences, Frederic, MD) in a total reaction volume of 20 μL. The reaction mix was incubated at 95°C for 5 min for the initial denaturation step, and then 40 cycles of the following steps: 10 s at 95°C, 20 s at 55°C, and 20 s at 72°C. Sequence of real time-PCR primers are presented in Table 2.1. Abundance of mRNA for each gene was calculated after its cycle threshold (Ct) was normalized to the Ct for 18S using the ΔΔCt method. Samples were analyzed in at least 6 replicates.

Western blot

Cells were initially washed twice with cold 1 × PBS after which they were suspended in 1 × radio-immunoprecipitation assay (RIPA) buffer [10% Nonidet P-40 (USB Co., Cleveland, OH), 0.5 M Tris-HCl (Ameresco, Solon, OH), 2.5% Deoxycholic acid (Sigma-Aldrich, St. Louis, MO), 1.5 M HCl, and 10 mM EDTA (Sigma-Aldrich)] supplemented with commercial protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Protein concentrations were determined by the bicinchoninic acid assay (BCA assay, Thermo Fisher Scientific, St. Louis, MO). Equal amounts of protein were loaded on 10% SDS polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc., Richmond, CA). Thereafter, membranes were blotted with respective primary antibodies: anti-β-actin (Cell Signaling Technology,

Danvers, MA), anti-heat shock protein (HSP) 70 (Cayman Chemicals, Ann Arbor, MI), anti-peroxisome proliferator-activated receptor γ (PPARγ) and anti-HSP90 (Cell Signaling Technology), anti-phosphoenolpyruvate carboxykinase 1 (PCK1 or PEPCK-C, Abcam Inc., Cambridge, MA), anti-glycerol kinase (GK, Bioss Inc., Woburn, MA), and anti-adiponectin antibody (Xeno Diagnostics, Indianapolis, IN, USA). Samples were analyzed in at least six replicates.

Respective secondary antibodies were used [horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit IgG, Cell Signaling]. Membranes were developed with Immobilon HRP substrate (Millipore, Billerica, MA) and exposed to autoradiographic film (Santa Cruz Biotechnology, Dallas, TX). Band intensities were quantified with a Kodak 1 D 3.6 software (Eastman Kodak, Rochester, NY).

Triglyceride (TAG) content determination

Total cellular lipid was extracted with isopropanol for 30 min at room temperature. Thereafter, TAG content was determined using a commercially available TAG determination kit (Sigma-Aldrich). Triglyceride content was normalized to cellular protein content (measured with the BCA assay).

Measurement of cellular ATP content

Concentration of ATP was measured in total cellular lysate recovered with 1 × RIPA buffer. Levels of ATP were measured with an ATP determination kit (Thermo Fisher Scientific). Cellular ATP amount was normalized to cellular protein content (measured through BCA assay).

Leptin ELISA

Cell culture medium was collected on d 6 of differentiation from adipocytes in both control and HS treatments. Leptin level was analyzed in duplicate wells with a pig leptin ELISA kit (CUSABIO, Wuhan, China) according to the manufacturer's instruction. The minimum detectable limit of this assay was 0.25 ng/ml. Intra-assay precision: CV%<15%.

Statistical analyses

Data were analyzed by ANOVA with GLM procedure and mean separation by Tukey analysis according to the procedures of SAS (SAS Inst. Inc., Cary, NC). Day and temperature were considered as main effects and day × temperature as interaction. Box-Cox transformation procedure was used when residuals were not normal. Differences were considered significant with $P < 0.05$, and P-values between 0.05 and 0.10 were considered as showing a strong tendency of significance. Values in the text are means ± SEM.

2.4 Results

Regulation of adipogenesis by HS

In order to test whether HS directly regulates adipocyte differentiation in the absence of other interfering in vivo factors, preadipocytes were subjected to differentiation over a 9-day period after which they were fully differentiated. Images from Oil red O staining, which stains total lipids, shows increased lipid staining of cells differentiated under HS compared to control conditions (Figure 2.1A). This is supported by the increased ($P < 0.0001$) content of TAG in cells differentiated in HS compared to control conditions (approx. 3 and 1.6-fold greater) on day 6 and 9 of differentiation, respectively (Figure 2.1B). mRNA levels of key adipogenic transcription factors, *PPARγ* and CCAAT/enhancer binding proteins α (C/EBPα), were differentially regulated by HS such that HS did not affect the mRNA levels of PPARγ ($P > 0.05$), but led to an increase ($P < 0.0001$) in the mRNA levels of C/EBPα relative to the control conditions (Figure 2.2B). PPARγ protein levels also shows that treatment did not affect its protein abundance (Figure 2.2C). Protein abundance of C/EBPα could not be determined due to the lack of a good antibody. It is unclear whether C/EBPα is regulated by the treatment at the protein level.

Heat stress promotes increased mRNA levels of fatty acid transport and glyceroneogenic genes

mRNA levels of several other genes implicated in increased TAG storage such as fatty acid synthase (*FAS*), LPL, adipocyte protein 2 (*FABP4 or aP2*), fatty acid translocase 36 (*CD36*), fatty acid transport protein 6 (*FATP6*) and glucose transporter protein type 4 (*GLUT4*) were presented significantly induced by HS compared to control (Figure 2.3). Unlike FATP6, mRNA

levels of fatty acid transport protein 4 (*FATP4*) was not different between control and HS condition (Figure 2.3 E and F). mRNA levels of PCK1 and GK was also measured. These are key enzymes involved in glyceroneogenesis, a pathway for making the glycerol backbone of TAG. Heat stress increased ($P < 0.0001$) the transcript levels of both PCK1 and GK (Figure 2.4 A and 4B), and their protein abundance (Figure 2.4 C and D).

Increased gene and protein level of adipokines in HS

Leptin and adiponectin play major paracrine, endocrine and autocrine roles in the regulation of metabolism (Lee and Shao, 2014; Blüher and Mantzoros, 2015), and may be implicated in the alteration of metabolism observed in pigs under HS. In order to test this hypothesis, we measured both leptin and adiponectin mRNA levels. mRNA levels of both adipokines were higher ($P < 0.05$) in HS compared to control conditions (Figure 2.5 A-E), suggesting that they may play critical roles during HS.

Heat stress promotes mRNA and protein level of *HSPs in pig adipocytes*

Heat shock proteins, molecular chaperones stablizing the damaging effects of HS, were also quantified. The mRNA levels of multiple HSPs (HSP27, HSP60, HSP70 and HSP90) were greater ($P < 0.0001$) in HS compared to control (Figure 2.6). Western blot analysis of HSP70, the major cytoplasmic HSP, and HSP90, also indicated that HS increased their protein abundance (Figure 2.6 E-F). Therefore, the induction of HSPs plays a role in the adaptive response against mild HS in pig adipocytes.

Heat stress leads to increase in cellular ATP level

Mild HS is able to activate fight-or-flight reactions which need the cellular ATP pool as energy source. Therefore, we measured cellular ATP concentrations in the differentiating adipocytes. As expected, total cellular ATP decreased with the progression of differentiation (Figure 2.7). However, cells in HS had greater ($P < 0.001$) cellular ATP than those in control temperature.

2.5 Discussion

Pigs have been known to conserve adipose tissue mass during HS (Collin et al., 2001; Kouba et al., 2001), resulting in increased adiposity, especially during the hot summer months. Previous studies have implicated the upregulation of LPL (Kouba et al., 2001) and FAS activities (Pearce et al., 2013a) in adipose tissue of pigs under mild HS condition. Increased LPL activity promotes greater hydrolysis of triglycerides in serum lipoproteins into fatty acids and glycerol, and these are then re-esterified into triglycerides in adipocytes, resulting in increased adipose tissue mass. Both LPL and FAS are transcriptionally regulated (Wolf et al., 1994; Zechner et al., 2000; Wang and Eckel, 2009) by insulin action, and this suggests that the increased mRNA level of these genes in HS may be tied to the elevated insulin concentrations observed in pigs under HS (Pearce et al., 2013a). However, a dominant role for elevated insulin is questionable (Williams et al., 2013; Pearce et al., 2014), given that HS in pigs is not always associated with elevated circulating serum insulin concentration, suggesting that additional mechanisms may be involved. In this study, we have applied an in vitro model of adipocyte differentiation to determine the direct effects of HS on porcine adipose tissue metabolism. This model provides a tool for direct investigation of effects of the elevated temperatures without the complications of in vivo factors such as variable insulin, glucocorticoids or catecholamine concentrations, or the potential involvement of other tissues.

Results from the current study indicate that HS stimulates adipogenesis in pig adipocytes and it is accompanied by increased TAG storage. Multiple genes that are involved in increased adipogenesis are induced by HS. Increased mRNA level of adipogenic transcription factor, C/EBPα, suggests that it may be implicated in the increased adipogenesis under HS. However, the mRNA and protein abundance of PPARγ were not affected by HS. This indicates that HS may not directly regulate its mRNA level. In addition, genes that are involved in increased fatty acid uptake (aP2, CD36, LPL, FATP6), de novo fatty acid synthesis (FAS), glucose uptake (GLUT4), and glyceroneogenesis (PCK1 and GK) were induced. The induction of these genes in HS may indeed reflect an increased cooperativity between C/EBPα and PPARγ during HS, because most of these genes are under transcriptional regulation by C/EBPα and PPARγ (Lin and Lane, 1994; MacDougald and Lane, 1995). Increased mRNA level of the fatty acid transporters, CD36 and FATP6, agrees with the expected increasing in free fatty acid availability, presumably

from an increased LPL activity. To support this idea, there is evidence that FATP6 and CD36 co-localize to enhance long chain fatty acid uptake, an interaction that is not seen between CD36 and FATP4 (Gimeno et al., 2003). Increased mRNA level of these fatty acid transporting genes may partly explain the reduction in serum fatty acid concentration in pigs under HS (Pearce et al., 2013a).

Induction of glyceroneogenic genes, PCK1 and GK, also provides another mechanistic explanation for the increased TAG storage in adipose tissue during HS. With the increased influx of free fatty acid into adipocytes, increased esterification of these acids is warranted to prevent lipotoxicity (Kawai et al., 2001). Both PCK1 and GK are directly involved in glyceroneogenesis in adipocytes, leading to the production of glycerol backbone that is critical for fatty acid condensation via glycerol-3-phosphate (Reshef et al., 2003). It is quite interesting that, compared to unstimulated mouse white adipocytes (Guan et al., 2002; MacDougald and Burant, 2005), pig adipocytes express significant amount of GK, and its mRNA and protein is inducible by HS. Therefore, both PCK1 and GK may play important roles in the increased lipid storage in pig adipocytes during HS.

Increased mRNA and protein level of adipokines (leptin and adiponectin) in adipocytes under HS is consistent with the report of others that HS stimulates secretion and expression of leptin and adiponectin in 3T3-L1 adipocyte (Bernabucci et al., 2009) and mice (Morera et al., 2012). Leptin, an adipokine that is expressed exclusively in adipose tissue, is a lipostat that plays a major role in appetite control through a feedback effect on key areas of the hypothalamus, ultimately leading to supersession of feed intake (Minokoshi et al., 2002). As we have shown previously, leptin administration results in reduction of feed intake in pigs (Ajuwon et al., 2003). A major adaptation to HS in pigs is the reduction in feed intake (Pearce et al., 2013a). Therefore, it is conceivable that elevated leptin concentration from adipocytes in pigs under HS may play an anorexigenic role in the animals leading to suppression of feed intake (Minokoshi et al., 2002).

Increased mRNA and protein level of HSP (HSP27, HSP60, HSP70 and HSP90), supports the hypothesis that non-lethal HS will result in appropriate adaptive response directly in pig adipocytes, comparable to increased tissue mRNA and protein level of HSP in pigs under HS (Pearce et al., 2013b). Although HSP are known to be involved in restoring cellular functions and enhancing cellular survivability during heat and other stresses (Horowitz and Robinson, 2007), their role in the increased lipid storage in adipocytes under HS is unknown. Heat shock protein 27 is an actin polymerization factor and its phosphorylation status promotes the polymerization of actin under HS conditions (Lavoie et al., 1995; Guay et al., 1997). Mechanistically, PPARγ could activate HSP22 which is responsible for activating HSP27 (Sun et al., 2004; Hamza et al., 2009). Furthermore, it is known that HSP90 acts as a chaperone for PPARγ, leading to increase its stability under stress (Nguyen et al., 2013), and there is evidence that HSP90 may be involved in the enhancement of the phosphoinositide-3-kinase–protein kinase B/Akt (PI3K-PKB/Akt) pathway under HS (Sato et al., 2000), a pathway that is involved in the stimulation of glucose uptake and GLUT4 translocation in adipocyte (Kohn et al., 1996). It is also known that HS stimulates HSP70 accumulation on adipocyte lipid droplets to stabilize the droplet monolayer, or chaperone denatured proteins for subsequent refolding (Jiang et al., 2007).

Whether HSP are involved in other functions to increase TAG storage in adipocytes is unknown. Nevertheless, based on their established function, induction of HSP in the adipocytes would be expected to serve a protective role, preventing the side effects of HS, such as ensuring appropriate protein folding, preventing protein aggregation, and refolding them into functional native states.

Heat stress response is an energy demanding process, with greater ATP requirement for increasing mRNA and protein level and activity of HSP. The reduction in cellular ATP with the progression of adipocyte differentiation in this study is consistent with earlier reports showing reduced cellular ATP concentration with adipocyte differentiation (Luo et al., 2008). However,

HS appears to preserve cellular ATP compared to cells at 37^{o}C. This agrees with the findings of Jayakumar et al. (1999) in myocytes that HS could help preserve cellular ATP level through alteration in energy metabolism profile, perhaps leading to increased production of ATP to support the HS response.

In conclusion, evidence is provided herein that HS directly leads to increased adipogenesis in porcine adipocytes. The mechanism of this response may include increased mRNA level of transcription factor C/EBPα, and increased mRNA level of fatty acid transport and glyceroneogenic genes. Increased leptin in adipocytes under HS may partly explain the reduction in feed intake observed in pigs under HS. These direct observations in adipocytes offer useful insights into the metabolic response in pig adipose tissue during HS, and provide plausible mechanisms underlying the increased adipose tissue mass long observed in pigs under HS.

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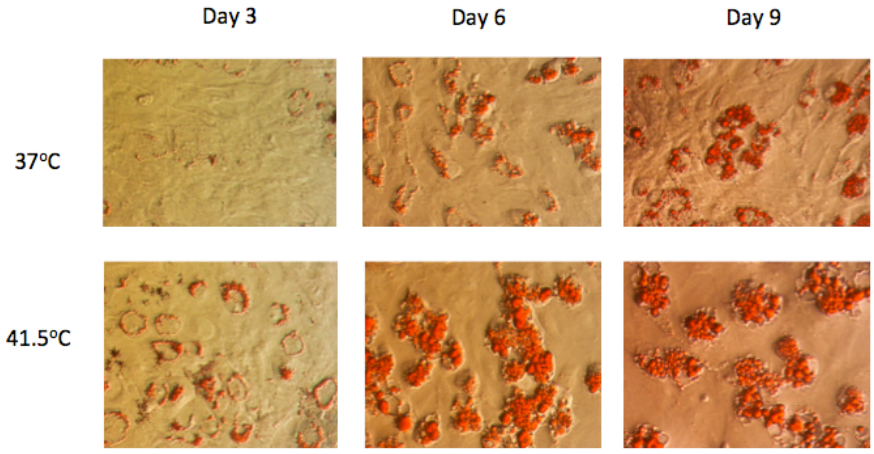
Table 2.1 Primer sequences for real time-PCR.

Gene[1]	Forward	Reverse
CD36	5'-ATC GTG CCT ATC CTC TGG-3'	5'-CCA GGC CAA GGA GGT TAA-3'
FATP4	5'-CAT TGT GGC TCA GCA GGT TA-3'	5'-CAG GCT AGG GGT CAA ATC AA-3'
C/EBPα	5'-TGG ACA AGA ACA GCA ACG AG -3'	5'- TTG TCA CTG GTC AGC TCC AG-3'
aP2	5'-TGG TAC AGG TGC AGA AGT GG -3'	5'-ATT CTG GTA GCC GTG ACA CC -3'
HSP70	5'-TTC GTG GAC AGA AGC CAC AG -3'	5'-TTG CTA GGA TCT CCA CCC GA -3'
FAS	5'-AGT TTG TGA TGG AGA ACA CGG CCT -3'	5'-TGT TCA CAC GTG GTG CAA GGG TTA -3'
GK	5'-CGC TGA GGA AAG TGA AAT CCG -3'	5'-TCG CGT CTT TGG AAT CTA CGA -3'
PCK1	5'-CCC TGC CTT TGA AAA AGC CC -3'	5'-GGA GAT GAT TTC TCG GCG GT -3'
18S	5'-ATC CCT GAG AAG TTC CAG CA-3'	5'-CCT CTT GGT GAG GTC GAT GT-3'
PPARγ	5'-GCC CTT CAC CAC TGT TGA TT-3'	5'-GTT GGA AGG CTC TTC GTG AG-3'
LPL	5'-ATT CAC CAG AGG GTC ACC TG-3'	5'-AGC CCT TTC TCA AAG GCT TC-3'
Leptin	5'-TTG GCC CTA TCT GTC CTA CG-3'	5'-GTG ACC CTC TGT TTG GAG GA-3'
HSP27	5'-GAG CTG ACG GTC AAG ACC AA-3'	5'-AAT GAA GCC GTG CTC ATC CT-3'
HSP90	5'-GTC GAA AAG GTG GTT GTG TCG-3'	5'-TTT GCT GTC CAG CCG TAT GT-3'
GLUT4	5'-GAA GGA AGA AGG CAA TGC TG-3'	5'-GAG GAA CCG TCC AAG AAT GA-3'
Adiponectin	5'-TTT CTG GGC CCA CTG TGT TT-3'	5'-GGT TTT GCA TTG CAG GCT CA-3'
HSP60	5'-AGA TGC CCT GAA TGC GAC AA-3'	5'-TGA CTC CAA GGC TGG AAT GC-3'
FATP6	5'-TTC TTC GGCTAT GCT GGC AA-3'	5'-TGG ACC ATTAGG TCT CCG GT-3'

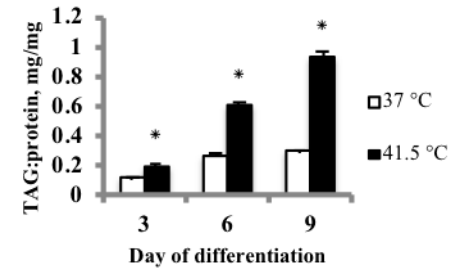
[1]aP2: fatty acid binding protein 2; CD36: fatty acid translocase 36; C/EBP α: CCAAT/enhancer binding proteins α; FATP4: fatty acid transport protein 4; FATP6: fatty acid transport protein 6; FAS: fatty acid synthase; GLUT4: glucose transporter protein type 4; GK: glycerol kinase; HSP: heat shock protein; LPL: lipoprotein lipase; PCK1: phosphoenolpyruvate carboxykinase 1; PPARγ: peroxisome proliferator-activated receptor γ.

Figure 2.1 Heat stress (HS) stimulates adipogenesis in adipocytes.

A

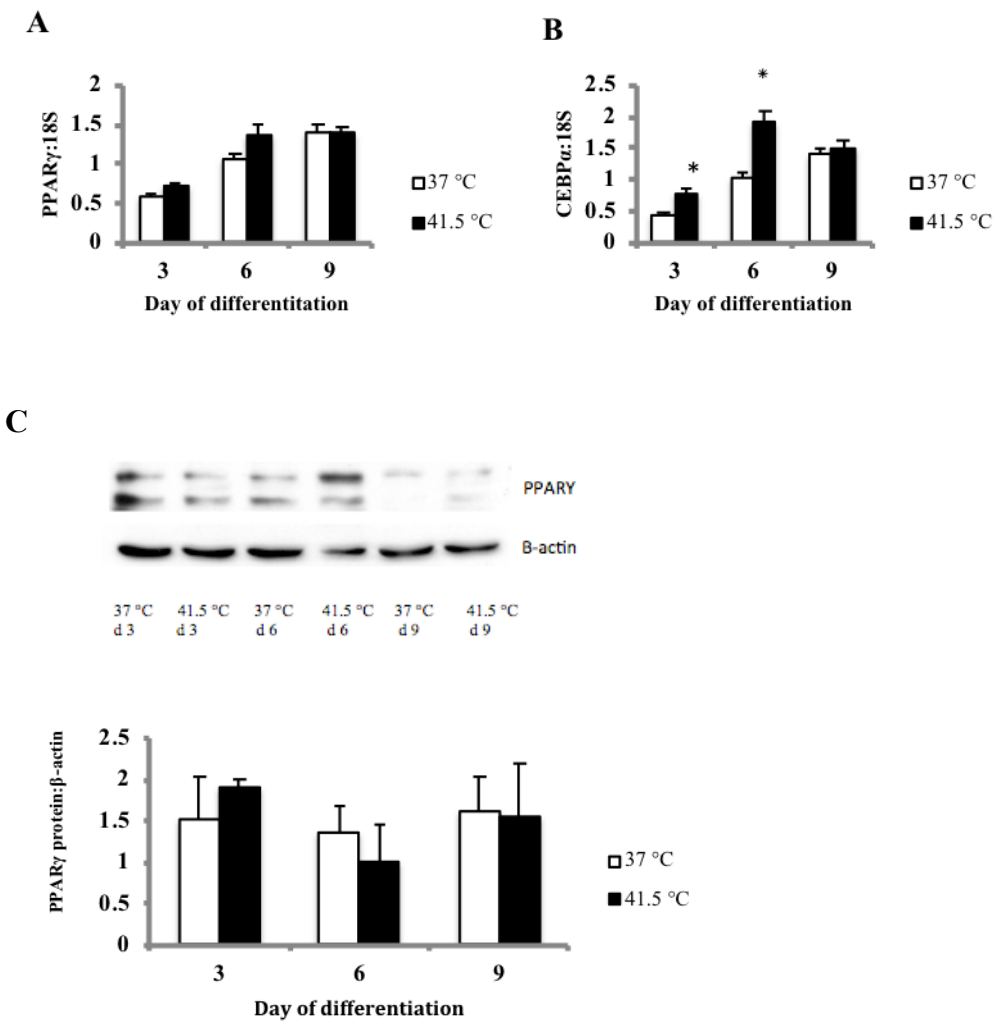


B



(A) Oil Red O staining for lipids in pig adipocytes on d 3, 6, and 9 of differentiation (40 ×) at 37°C and 41.5°C temperatures. (B) Triglyceride (TAG) concentration was measured in pig adipocytes under normal conditions (37°C) and HS condition (41.5°C) on d 3, 6, and 9 respectively. Bars represents means ± SEM of at least 6 different replicates. * indicates mean difference between HS and control at $P < 0.05$ within day of differentation.

Figure 2.2 Expression of genes related to adipogenesis in differentiating adipocytes under normal (37°C) and heat stress (41.5°C) conditions.



(A) Peroxisome proliferator-activated receptor γ (PPARγ) gene expression. (B) CCAAT/enhancer binding proteins α (C/EBPα) gene expression. (C) PPARγ immunoblotting (upper panel) and quantification (lower panel). Bars represents means ± SEM of at least 6 different replicates. * indicates mean difference between HS and control at $P < 0.05$ within day of differentiation.

Figure 2.3 Expression of genes involved in fatty acid transport and binding, and glucose transport in adipocytes under normal condition (37°C) and heat stress condition (41.5°C) on d 3, 6, and 9 respectively.

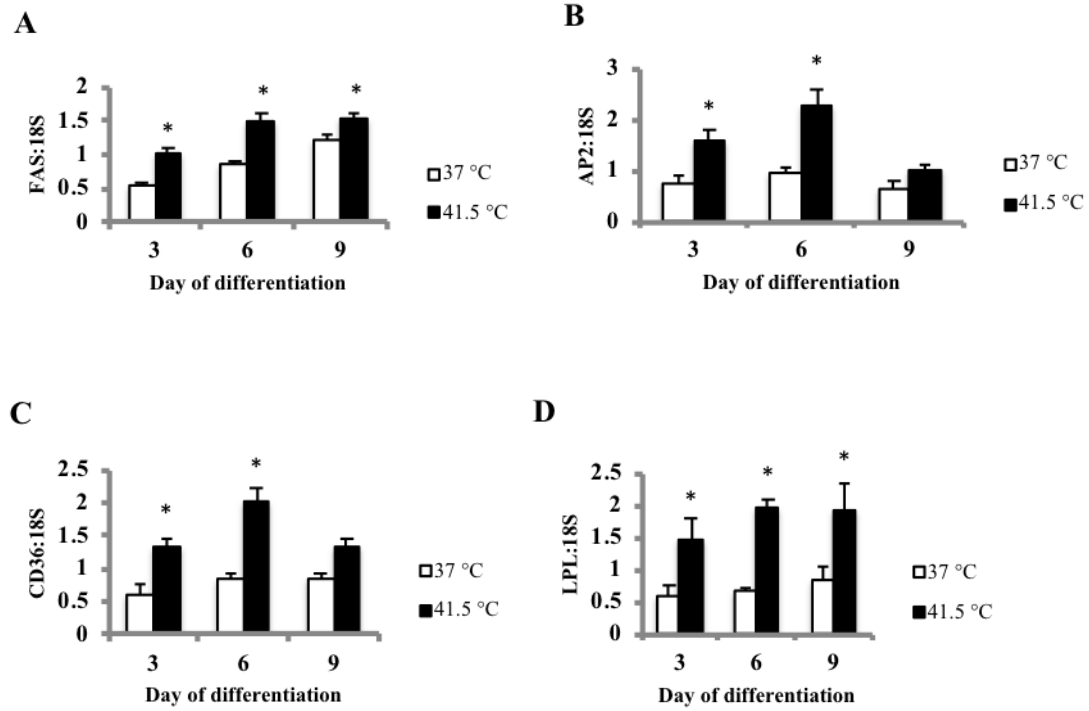
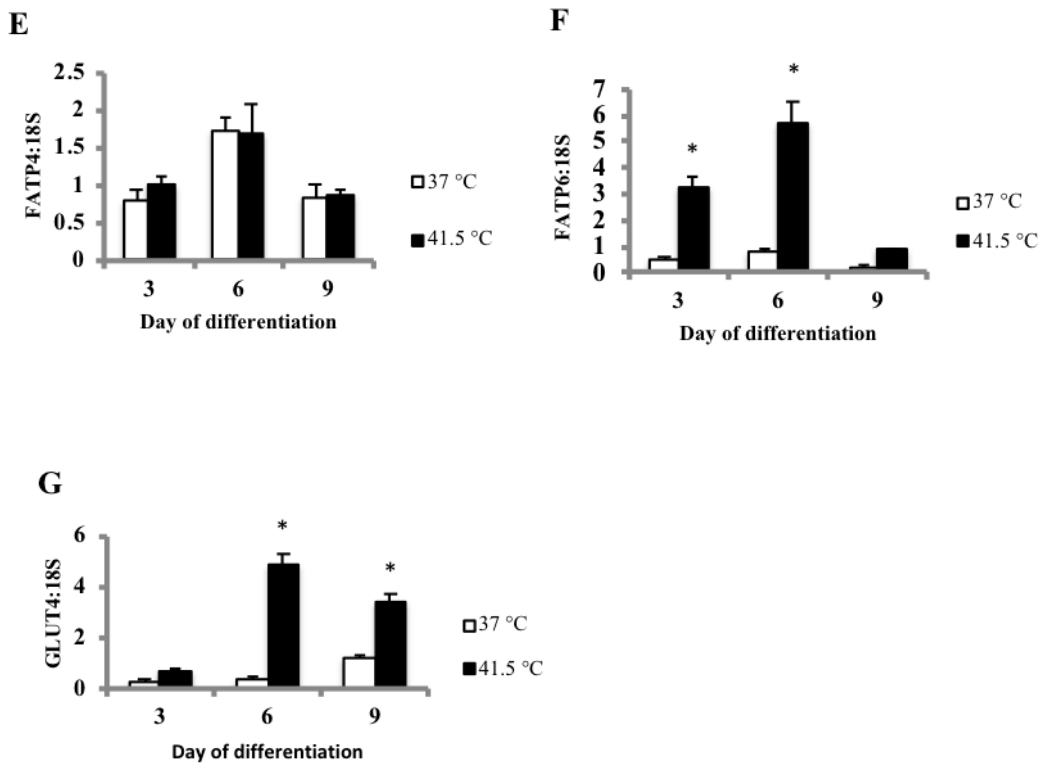
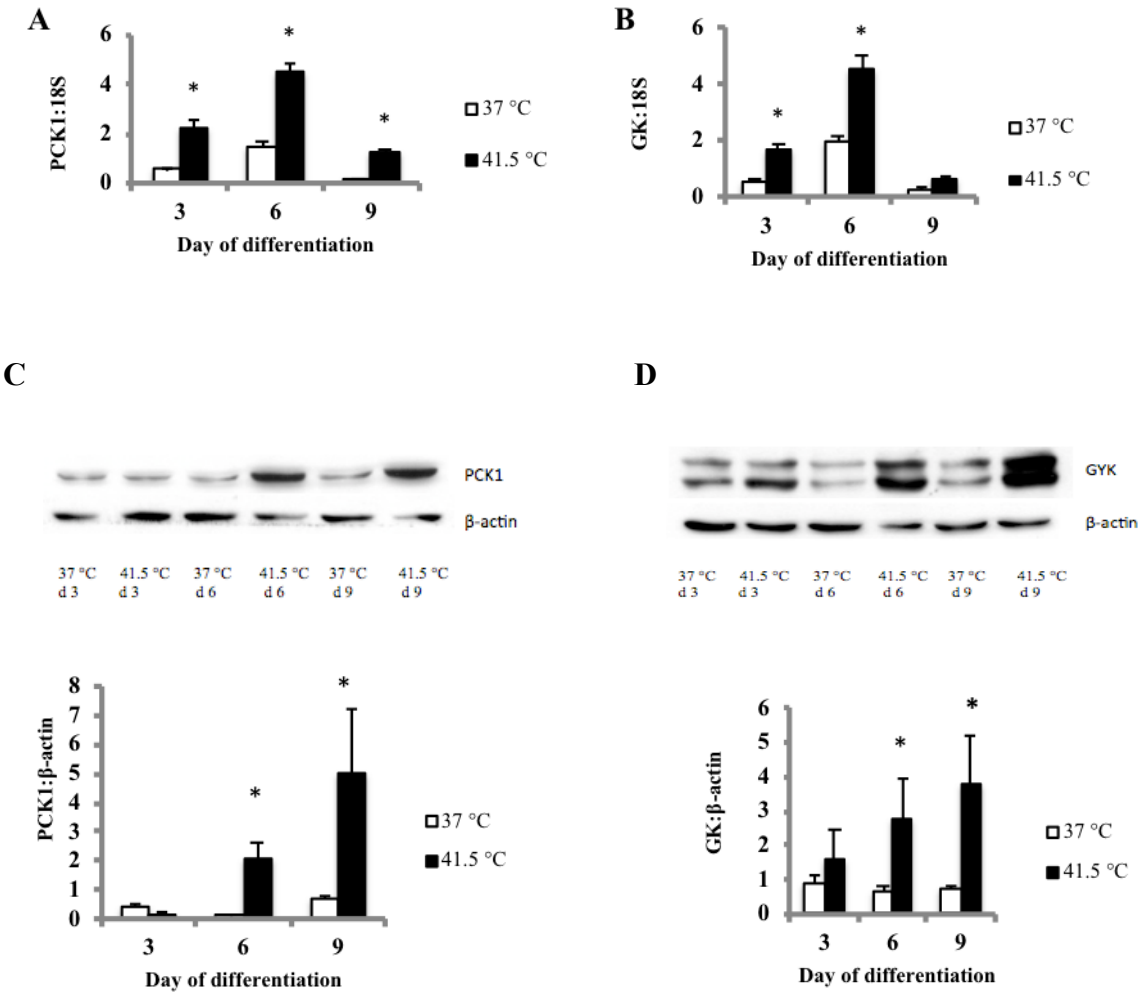


Figure 2.3 Contiuned



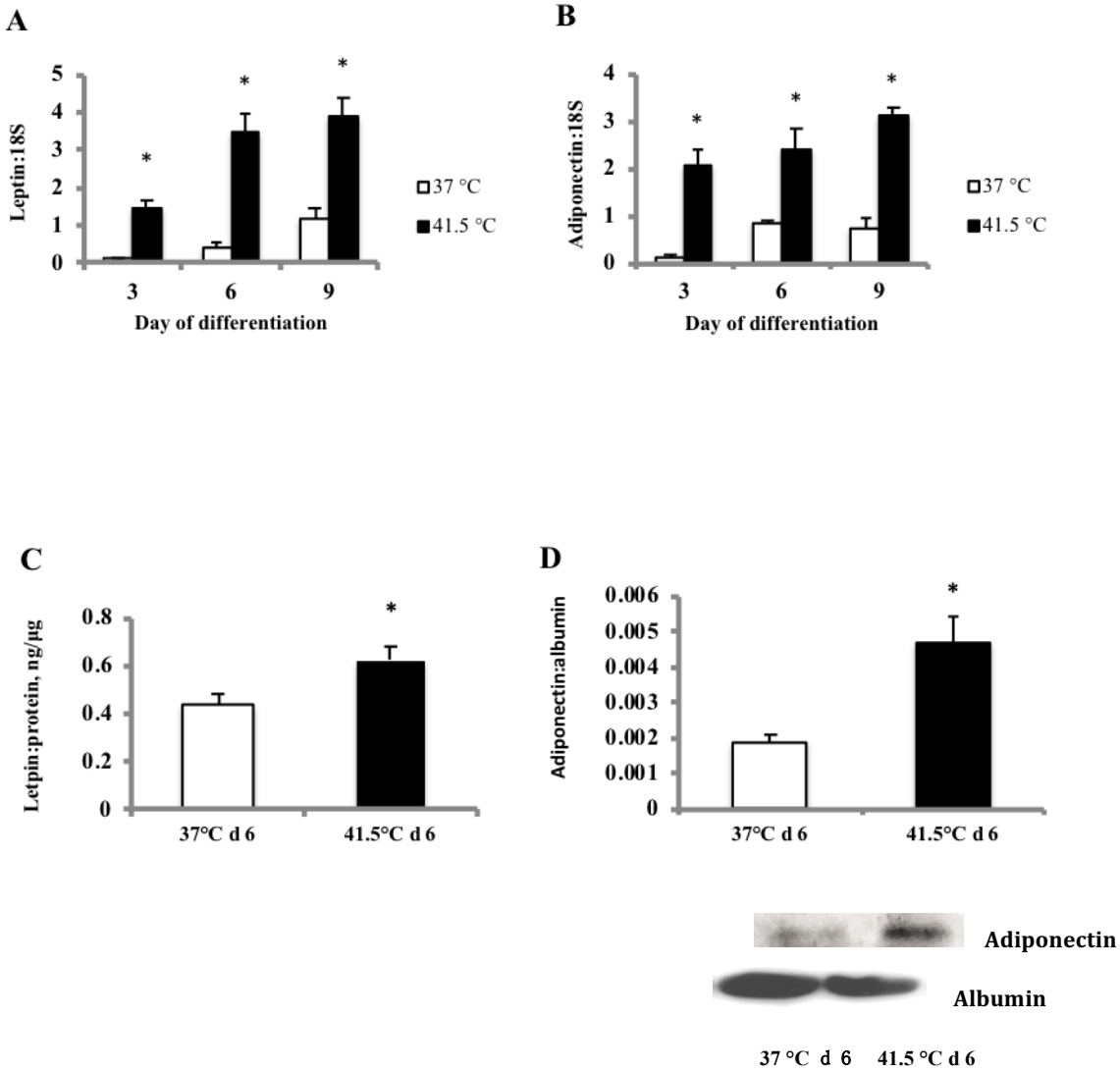
Genes were measured by real time-PCR. (A) Fatty acid synthase (FAS). (B) Adipocyte protein 2 (aP2). (C) Fatty acid translocase 36 (CD36). (D) Lipoprotein lipase (LPL). (E) Fatty acid transport protein 4 (FATP4). (F) Fatty acid transport protein 6 (FATP6). (G) Glucose transporter protein type 4 (GLUT4). Bars represents means ± SEM of at least 6 different replicates. * indicates mean difference between HS and control at $P < 0.05$ within day of differentiation.

Figure 2.4 Heat stress increases abundance of genes involved in glyceroneogenesis in adipocytes.



(A) Phosphoenolpyruvate carboxykinase 1 (PCK1 or PEPCK-C) and (B) glycerol kinase (GK) mRNA levels were measured by real time-PCR. (C) and (D) are immunoblots for PCK1 and GK respectively. Bars represents means ± SEM of at least 6 different replicates. * indicates mean difference between HS and control at $P < 0.05$ within day of differentiation.

Figure 2.5 Heat stress stimulates adipokines mRNA and protein level in pig adipocytes.



(A) leptin and (B) adiponectin mRNA abundance was measured by real time-PCR in adipocytes under normal (37°C) and heat stress (41.5°C) conditions. (C) Media leptin was measured by ELISA. (D) Media adiponectin was measured by western blotting with quantification. Bars represents means ± SEM of at least 6 different replicates. * indicates mean difference between HS and control at $P < 0.05$ within day of differentiation.

Figure 2.6 Heat stress stimulates heat shock protein (HSP) mRNA and protein level in pig adipocytes.

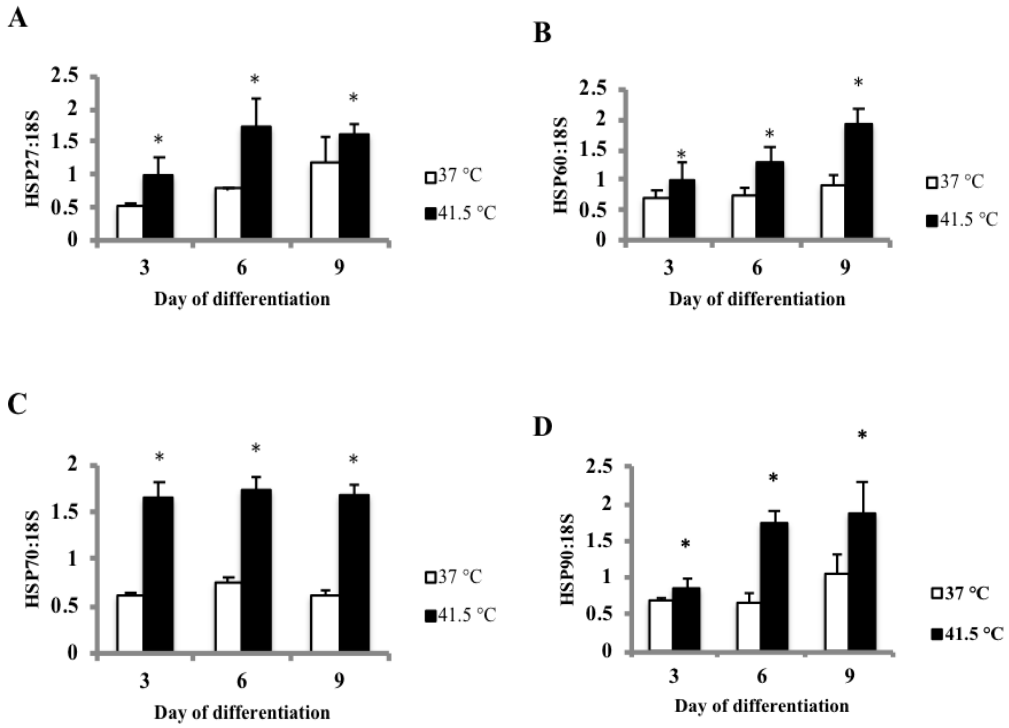
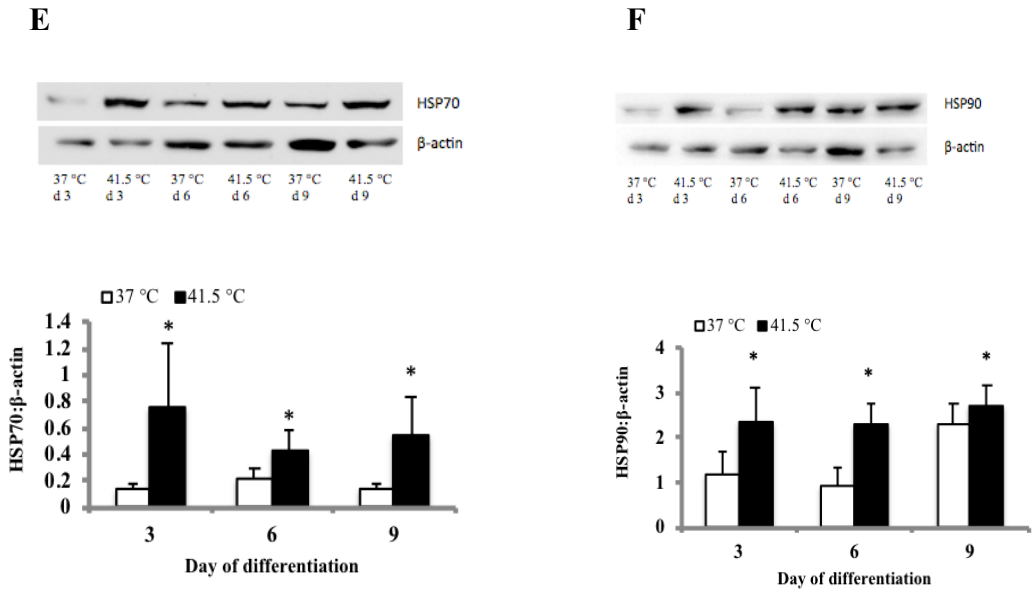
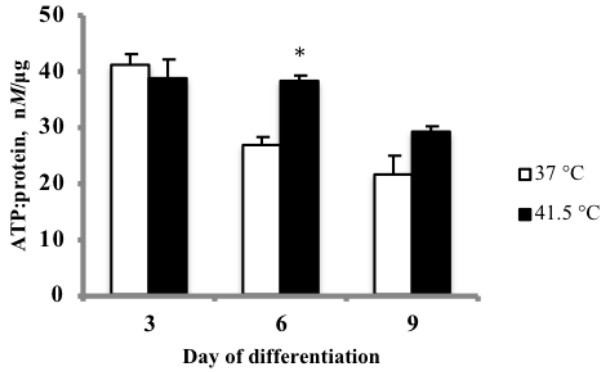


Figure 2.6 Contiuned



Transcript levels of HSP 27(A), HSP60 (B), HSP70 (C) and HSP90 (D) were measured in pig adipocytes under normal (37°C) and heat stress (41.5°C) conditions using real time-PCR. (E) HSP70 and (F) HSP90 protein levelwas determined by western blot. Bars represents means ± SEM of at least 6 different replicates. * indicates mean difference between HS and control at $P <$ 0.05 within day of differentiation.

Figure 2.7 Heat stress leads to increase cellular ATP concentration.



ATP level was measured in pig adipocytes under normal (37°C) and heat stress (41.5°C) conditions. Bars represents means ± SEM of at least 6 different replicates. * indicates mean difference between HS and control at $P < 0.05$ within day of differentiation.

CHAPTER 3. HEAT STRESS IN PIGS IS ACCOMPANIED BY ADIPOSE TISSUE-SPECIFIC RESPONSES THAT FAVOR INCREASED TRIGLYCERIDE STORAGE

3.1 Abstract

Heat stress (HS) negatively affects all aspects of performance in pigs. Although certain tissue-specific responses in the liver, skeletal muscle, and intestine are known, there is paucity of information on responses within the adipose tissue. Therefore, the objective of this study was to delineate adipose tissue responses during HS in pigs. Thirty crossbred (Ossabaw × Duroc × Landrace) pigs were assigned to 3 treatments for 7 d. Treatments were 1) control and libitum fed (CON) with room temperature set at 20°C ± 1°C, 2) pair fed (PF) with room temperature as the CON treatment but pair fed to HS pigs, and 3) HS with room temperature 35°C ± 1°C and ad libitum access to feed. Experiment lasted one week. Compared with CON pigs, HS pigs had decreased feed intake and elevated skin temperature and respiration rate ($P < 0.01$). Blood urea nitrogen was higher ($P = 0.01$) in HS pigs compared with CON pigs only in males. In both subcutaneous and mesenteric adipose tissue, mRNA abundance of phosphoenolpyruvate carboxykinase (PCK1) was more elevated ($P < 0.01$) in HS groups compared with the CON and PF groups. Heat stress also caused increased heat shock protein 70 (HSP70; $P = 0.067$) and CCAT/enhancer-binding homologous protein (CHOP) content ($P < 0.05$) in the mesenteric fat compared with the CON treatment. In conclusion, induction of PCK1 expression in adipose tissue by HS suggests elevated glycroneogenesis might be involved in the increased fat storage in pigs under HS.

Keywords: adipose tissue, CHOP, heat stress, PCK1 or PEPCK, pig

3.2 Introduction

Heat stress in pigs costs the US swine industry millions of US dollars annually (St-Pierre et al., 2003). Pigs may be more susceptible to heat stress due to lack of functional sweat glands on their skin surface (Curtis, 1983), and also because pigs have substantial subcutaneous fat layer that serves as a barrier against effective heat dissipation. Heat stress leads to behavioral and metabolic (homeostatic and homeorhetic) adaptations in animals (Bernabucci et al., 2010) aimed at increasing tolerance and survivability. These changes include reduction in feed intake and voluntary movement, increased respiration rate and water consumption, and in the case of the pig, wallowing or other forms of recreational water use. Heat stress in the pig also results in preferential retention of adipose tissue through upregulation of activities of enzymes such as lipoprotein lipase (Christon, 1988; Kouba et al., 2001). We recently demonstrated the existence of cell autonomous response to heat stress in pig adipocytes leading to increased adipocyte lipid storage (Qu et al., 2015). Notably, we observed induction of genes involved in glyceroneogenesis, phosphoenolpyruvate carboxykinase (PCK1 or PEPCK-C) and glycerol kinase (GK). This confirms that heat sensing occurs at the adipocyte to increase fatty acid esterification through induction of glyceroneogenesis. However, whether similar mechanisms are involved in heat stress response in adipose tissue in vivo is unknown.

Therefore, the objective of this work was to determine adipose tissue-specific responses to heat stress may provide the mechanistic explanation of the increased lipid storage in heat-stressed pigs. We also hypothesized that genes involved in glyceroneogenesis might play important roles in vivo in the increased lipid storage in pigs under chronic heat stress.

3.3 Materials and methods

Animals and experimental design

The Purdue Animal Care and Use Committee approved protocols and all animal care described in these experiments. Thirty cross-bred pigs (Ossabaw × Duroc × Landrace) pigs at 4 months of

age were assigned to three treatments: control and libitum fed (CON), pair-fed (PF) and heat stress (HS). There were 10 pigs (5 males and 5 females) in each treatment. The temperature in the CON treatment was set at 20 °C ± 1 °C and relative humidity varied between 53% -78%. The room temperature in the HS treatment was set at 35 °C ± 1 °C and relative humidity allowed to vary freely from 45% - 65%. Pigs were allowed ad libitum feed intake. The pair-fed (PF) pigs were held at the same room temperature and relative humidity as the CON group, and pair fed to the feed intake of pigs in the HS treatment. All pigs had free access to water.

Animals were allowed to adjust to their pens for 7 d at the control temperature before the start of the experiment. During the adjustment period, ad libitum levels of feed intake of pigs were determined. After the adjustment period pigs were allocated to the 3 treatments for 7 d. Feed intake was determined daily at 0800 h. Pair-fed pigs were fed twice daily at 0900 and 0500 h with an amount of feed that was equal to the previous day's FI in the HS treatment. The nutrient concentrations in the feed met or exceeded NRC (2012) requirements (Table 3.1).

Body weight and skin and rectal temperatures were recorded at the start and the end of the experiment. Feed intake and skin temperature were measured daily during the experiment. Skin temperatures were measured with an infrared noncontact digital thermometer (model WIC-276664; Walgreens, Deerfield, IL). Rectal temperatures were measured with a digital rectal thermometer (model KD-113; Walgreens). Respiration rate was measured 3 independent times on the last 3 d of the experiment and averaged. Blood was collected on d 0 and 7 of the experiment. Pigs were humanely sacrificed at the end of the experiment and tissue samples were collected and flash frozen in liquid nitrogen for further analyses. Although adipose tissue response was the major focus of the study, liver and muscle (longissimus dorsi muscle) tissue samples were also collected for speci c comparisons.

Analysis of mRNA Abundance

Expression levels of selected genes in adipose tissue, liver, and muscle were determined by real-time PCR. Total RNA was extracted using QIAzol lysis reagent (Qiagen Inc., Valencia, CA) and dissolved in nuclease-free water (Ambion, Austin, TX). Concentrations of RNA were determined with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE).

Integrity of RNA and genomic DNA contamination were checked by electrophoresis on 0.8% agarose gel. Ribonucleic acid was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Promega Corporation, Madison, WI). A PCR assay was conducted on a Bio-Rad MyiQ thermocycler (Bio-Rad Laboratories Inc., Temecula, CA) with the SYBR real-time PCR mix (Qiagen Inc.) in a total reaction volume of 20 μL. Sequences of real-time PCR primers are presented in Table 2. A melt-curve analysis was performed for each gene at the end of the PCR run. Expression level for each gene was calculated after its cycle threshold (Ct) was normalized to the Ct for 18S using the ΔΔCt method.

Western blot analysis

Tissues were homogenized in 1x radioimmunoprecipitation assay buffer (50 M Tris-HCl [AMRESCO LLC, Solon, OH], 0.25% deoxycholic acid [Sigma-Aldrich Corp., St. Louis, MO], 15 M NaCl, 10 mM EDTA [Sigma-Aldrich Corp.], and 0.1% Triton X [Sigma- Aldrich Corp.]) supplemented with commercial protease and phosphatase inhibitor cocktails (Sigma-Aldrich Corp.). Homogenates were then centrifuged at 10,000 × g for 10 min at 4°C to remove tissue debris. Protein concentrations were determined by the bicinchoninic acid assay method (Thermo Fisher Scientific Inc., St. Louis, MO). Equal amounts of protein were resolved on 10% SDS polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories Inc., Richmond, CA). Membranes were blotted with primary antibodies: anti-β-actin (Cell Signaling Technology, Inc., Danvers, MA), anti-heat shock protein (HSP) 70 (Cayman Chemical, Ann Arbor, MI), anti-PCK1 (Abcam Inc., Cambridge, MA), anti-adiponectin antibody (Xeno Diagnostics, LLC, Indianapolis, IN), and anti-CCAT/ enhancer-binding homologous protein (CHOP; Cell Signaling Technology, Inc.). Secondary antibodies were either horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit IgG (Cell Signaling Technology, Inc.). Chemiluminescent signals were developed with Immobilon HRP substrate (EMD Millipore, Billerica, MA) and captured by autoradiographic film exposure (Santa Cruz Biotechnology, Inc., Dallas, TX). Intensities of bands were analyzed with Kodak 1 D 3.6 software (Eastman Kodak Co., Rochester, NY).

Analysis of plasma metabolites

Total plasma triglyceride (TAG) concentration was determined using a commercially TAG determination kit (Sigma-Aldrich Corp.). Plasma FFA was measured using the Free Fatty Acids Half-Micro Test Kit (Roche Biosciences, Indianapolis, IN). Blood urea nitrogen (BUN) was measured with a BUN colorimetric detection kit (Arbor Assays, Ann Arbor, MI). Plasma glucose was measured with Autokit Glucose (Wako Diagnostics, Richmond, VA). All assays were done according to the manufacturers' instructions.

Insulin ELISA

Plasma insulin was measured using a Mercodia porcine insulin ELISA kit (Mercodia, Uppsala, Sweden). This assay has an intra-assay coefficient of variability of 3.1- 3.8% and an inter-assay variability of 1.5-3.9%. The assay was performed according to the manufacturer's instructions.

Statistical analyses

Data were analyzed by ANOVA with PROC MIXED procedure (SAS Inst. Inc., Cary, NC). Sex and treatment were considered as main effects and sex × treatment as interaction. Feed intake and skin temperature were measured daily. These variables were analyzed using repeated measures and day as the repeated effect. Box-Cox transformation procedure was applied when residuals were not normal. Differences were considered significant with $P < 0.05$ and were considered as showing a strong tendency of significance with P-values between 0.05 and 0.10. When main or interaction effects were significant, a Tukey mean comparison analysis was conducted to separate the means. Superscript letters indicate significant mean differences. Data are presented as means ± SEM.

3.4 Results

Animal performance

The HS treatment had a lower (approximately 40% lower) FI compared with the CON treatment ($P < 0.01$; Fig. 3.1A and 3.1B). By design, the FI of PF pigs was similar to that of pigs in the HS treatment. There was a significant treatment × sex interaction effect because males under HS,

although they experienced an immediate reduction in FI after the HS treatment, progressively recovered over time. However, females did not have a similar pattern of gradual recovery. Heat-stressed pigs had increased skin temperature (+6°C) and respiration rate (2 fold; Fig. 3.1C, 3.1D, and 3.1E) compared with CON and PF pigs ($P < 0.01$). Heat-stressed pigs also had a higher (+0.3°C) rectal temperature ($P < 0.05$) than CON and PF pigs, and these variables were higher in the CON pigs than in the PF pigs (Table 3.3).

Plasma metabolites

Plasma TAG and FFA concentrations were not affected by treatment ($P > 0.05$), but gilts had higher ($P < 0.01$) concentrations of both metabolites than boars (Fig. 2A and 2B). There was a significant ($P < 0.05$) treatment × sex interaction effect on BUN concentration. Under HS, BUN was higher in boars (1.4 fold) compared with those in the CON treatment, whereas there were no treatment differences in gilts (Fig. 3.2C). Although there were no treatment differences in serum insulin concentrations ($P > 0.05$), there was a tendency ($P = 0.097$) for a lower insulin concentration in gilts than boars. Similarly, glucose concentration was not influenced by treatment but was higher (1.9 fold) in boars than gilts ($P = 0.029$; Fig. 3.2D, 3.2E, and 3.2F).

Tissue specific regulation of mRNA of selected genes and proteins

Adipose Tissue. In the subcutaneous fat, treatment did not affect the mRNA abundance of HSP70 ($P > 0.05$). However, gilts had a higher (1.4 fold; $P = 0.04$) abundance of this gene than boars (Table 3.4). Interestingly, the content of HSP70 protein was higher ($P = 0.035$) in boars (1.8 fold) than in gilts in this depot (Fig. 3.3B). In the mesenteric fat, although the mRNA abundance of HSP70 was not affected by treatment (Table 3.5), there was a tendency ($P = 0.067$) for a higher content of HSP70 protein in the HS treatment than in the CON and PF treatments in the mesenteric fat (Fig. 3.4B). The mRNA abundance of PCK1 was elevated ($P < 0.01$) in the HS treatment compared with the CON treatment in both the subcutaneous (22 fold; Table 4) and mesenteric (11 fold) fat depots (Table 3.5). In addition, there was a tendency for an elevated PCK1 protein content in the HS treatment in the subcutaneous fat depot ($P = 0.09$; Fig. 3.3C) and a significant elevation ($P = 0.034$) in the mesenteric fat depot (Fig. 3.4 C). Sex effect was also significant, such that boars had a higher abundance of PCK1 mRNA in the subcutaneous (3 fold;

P = 0.023; Table 3.4) and mesenteric fat (3 fold; P = 0.025; Table 3.5) than gilts. There appears to be tissue- and sex-specific differences in the regulation of GK because its mRNA abundance was higher in boars in the HS treatment than in boars in the PF and CON treatments in the subcutaneous fat (1.6 fold; P = 0.048; Fig. 3.3A). However, HS led to a reduction (54%) in its mRNA abundance in gilts in mesenteric fat compared with the PF treatment (P = 0.011; Fig. 3.4A). There were no differences in the protein levels of GK in the adipose tissues, irrespective of sex or treatment (data not shown).

Fatty acid synthase (FAS) is another important gene involved in fatty acid synthesis and storage in adipocytes. There were no treatment differences in FAS mRNA abundance in the subcutaneous (Table 3.4) and mesenteric (Table 3.5) adipose depots, but there was a tendency (P < 0.06) for a higher abundance in the subcutaneous fat in gilts than in boars (Table 4) and there was a significantly higher (P < 0.01) abundance (1.4 fold) in gilts than in boars in the mesenteric fat (2 fold; Table 3.5). The mRNA abundance of lipoprotein lipase (LPL; 1.7 fold) and leptin (2.5 fold) were higher (P = 0.004 and P = 0.030, respectively; Table 3.4) in the HS treatment compared with the PF treatment only in the subcutaneous fat depot. Additionally, in the subcutaneous depot, abundance of glucose transport type 4 (GLUT4) mRNA was lower (50%; P = 0.016) in the PF treatment compared with the CON treatment and was lower in gilts than in boars in both subcutaneous (40% lower; Table 3.3) and mesenteric (70%; P < 0.01; Table 3.4) depots. Abundance of diacylglycerol O-acyltransferase 2 (DGAT2) mRNA was similarly lower (40%; P < 0.05) in gilts than in boars in both subcutaneous and mesenteric adipose depots (Tables 3.4 and 3.5). Abundance of fatty acid binding protein 2 (aP2) mRNA was higher (1.4 fold) in gilts than in boars in the subcutaneous adipose depot without any treatment effect (Table 3.4) but was not differentially expressed in the mesenteric depot (Table 3.5). Inflammatory cytokines, IL-6 and tumor necrosis factor a (TNF-α), were differentially regulated as well. In the subcutaneous tissue (Table 3.4), although abundance of IL-6 mRNA was higher in boars (1.8 fold; P < 0.05), that of TNF-α was higher in gilts (1.8 fold; P < 0.05) and there were no significant treatment differences. Expression of the 2 cytokines in the mesenteric depot was not affected by treatment. In the subcutaneous depot, CHOP protein content was higher in boars (1.9 fold) than in gilts (P < 0.01; Fig. 3.3D). Furthermore, the content of CHOP protein in the

mesenteric depot was higher (P = 0.019) in the HS treatment (2.5 fold) than in the CON treatment (Fig. 3.4D) without any effect of sex.

Liver specific responses

Selected responses were measured in the liver for comparative purposes with adipose tissue. Abundance of HSP70 mRNA was higher (P < 0.05) only in boars under HS than in boars in the CON treatment (4.5 fold) due to a significant (P < 0.03) treatment × sex interaction effect (Fig. 3.5A). However, there was neither treatment nor sex effects on HSP70 protein content (Fig. 3.5B). The mRNA abundance of PCK1 (Table 3.6) was not affected by either sex or treatment, although there was a tendency (P < 0.09) for a higher expression in gilts (Fig. 3.5C). In addition, GK mRNA abundance was not affected but FAS mRNA abundance was lower (P < 0.01) in gilts than in boars (Table 3.6). The level of CHOP protein was affected by treatment (P = 0.02; Fig. 3.5D). Pigs in the PF treatment had a higher content of CHOP protein than pigs in the CON treatment (7 fold) but it was similar to expression in pigs in the HS treatment.

Muscle specific responses

Selected responses were also measured in the longissimus dorsi muscle for comparison with adipose tissue. There was a significant (P = 0.04) treatment × day interaction on the mRNA abundance of HSP70 in the longissimus dorsi muscle (Fig. 3.6A). Although there was no treatment difference in gilts, HSP70 mRNA abundance was higher (6.5 fold) in the HS treatment than in the PF treatment (P = 0.044; Fig. 3.6A). However, at the protein level, HSP70 was not affected by either treatment or sex (Fig. 3.6B). Unlike adipose tissue, PCK1gene and protein abundance were not affected by either treatment or sex (Table 3.7; Fig. 3.6C). In addition, abundance of GK mRNA was similar across groups (Table 3.7). The content of CHOP protein was equally unaffected by either treatment or sex (Fig. 3.6D).

3.5 Discussion

Heat stress results in reduced nutrient intake in pigs and altered energy partitioning and carcass quality in pigs (Pearce et al., 2013). The physiological basis of this response is indeed very complex and animals gain more adipose tissue than energetically predicted under a HS environment (Yunianto et al., 1997; Kouba et al., 1999; Collin et al., 2001; Pearce et al., 2013). To understand adaptive changes in the pig that are independent of FI levels, we used a PF group to minimize the confounding effects of different FI between animals held in a HS environment versus those under thermoneutral condition. Therefore, comparison between the PF and the HS groups make the most biological sense. Because we were specifically interested in adaptive responses within the adipose tissue, the use of a crossbred line that included the Ossabaw genotype allowed us to use a genetic background with substantial adipose mass relative to terminal commercial crossbred lines. In addition, the Ossabaw pig is prone to insulin resistance and metabolic syndrome (Spurlock and Gabler, 2008), and it has been demonstrated that diabetic humans and rodents are more sensitive to HS and heat-related diseases (Semenza et al., 1999; Niu et al., 2003).

Heat-stressed pigs experienced modest increases in skin and rectal temperatures compared with the CON and PF pigs, and the effect of the elevated temperatures was reflected in the reduction in FI in the HS pigs. It is plausible that the inadequate nutrient intake during HS partly explains the lack of weight gain or weight loss observed under HS. Reduction in FI is a recognized adaptive response across multiple species (Collin et al., 2001; Baumgard et al., 2012) for decreasing metabolic heat production (Kouba et al., 2001). Nevertheless, HS results in unique metabolic adaptation that minimizes weight loss in animals in comparison with PF controls. In this study, although pigs in the PF treatment lost 0.64 kg BW, the HS pigs gained 1.05 kg BW at the end of the study. These differences in tissue accretion rates may be explained by differences in the lean/fat tissue deposition rates and maintenance costs between the HS and the PF groups (McDowell et al., 1969; Collins et al., 1980; Yahav, 2007; Baumgard and Rhoads, 2013; Pearce et al., 2013).

The reduction in FI in the HS group could be partly explained by the higher leptin expression in adipose tissue in the HS group. We had earlier demonstrated that leptin leads to suppression of FI in pigs (Ajuwon et al., 2003). The leptin response to HS in pig adipose tissue is also consistent with recent in vitro data in pig adipocytes (Qu et al., 2015) and in mice (Morera et al., 2012). Although HS induces an upregulation of the expression of orexigenic neuropepetides such as neuropeptide Y (Ito et al., 2015), an induction of leptin in adipose tissue may override the orexigenic effects of these peptides, and hence the observed reduction in FI during HS.

Therefore, a function of leptin may be to limit metabolic heat production during HS by suppressing FI. Unlike other studies in rodents (Bernabucci et al., 2009; Morera et al., 2012), HS did not result in elevated adiponectin expression in adipose tissue from HS pigs. Therefore, possible species differences might exist in the regulation of adiponectin expression under HS. Heat stress did not result in alteration in serum insulin concentration in this study. Data on the regulation of insulin concentration in pigs during HS is controversial. Although a few studies have found that HS results in higher basal and stimulated circulating insulin in pigs (Pearce et al., 2013) and ruminants (Baumgard and Rhoads, 2013), others did not detect basal insulin changes (Sanz Fernandez et al., 2015a). The mechanism by which HS might increase insulin secretion is quite intriguing because HS is accompanied by elevated catecholamine and cortisol secretions, and both hormones are known to suppress pancreatic insulin secretion (Doi et al., 1982). Therefore, the regulation of plasma insulin concentration during HS may be complex and warrants further investigation.

Glucose concentration was not significantly suppressed in HS pigs. It appears that, unlike ruminants, rats, and chickens in which serum glucose concentration is suppressed during HS (Mitev et al., 2005; Rahimi, 2005; O'Brien et al., 2010), pigs are able to maintain normoglycemia during HS. This is consistent with the findings of Pearce et al. (2013). Several factors may be responsible for the similar glucose concentrations across treatments. First, insulin

concentrations were similar in all treatments, perhaps exerting similar effects on tissue glucose uptake in all treatments. Second, the lack of treatment effect on glucose uptake may also be reflected in the similar expression of GLUT4 between the HS and the PF groups. The lack of effect of treatment on GLUT4 may be related to the similar insulin concentrations in treatments (Hadley, 2000). Protein catabolism is usually increased during chronic HS (Allen, 1988), and HS increased plasma BUN concentration in males compared with the CON and PF pigs. It is reasonable to surmise that under HS, there is increased muscle breakdown, and perhaps more so in males.

Apart from the elevated TAG and FFA in gilts, there was no treatment effect on serum concentrations of these metabolites. Although it is known that pigs on a low nutrient intake will mobilize adipose tissue TAG and increase plasma NEFA concentration (Vernon, 1992), it is possible, however, that the magnitude of the reduction in FI was not severe enough in the HS and the PF treatments or could be due to the duration of the HS treatment. Marple et al. (1974) demonstrated that acute HS led to an increase in serum NEFA concentration, and chronic HS induced decreased NEFA concentration compared with pair-fed thermal-neutral (PFTN; Sanz Fernandez et al., 2015b) in pigs. The regulation of lipid metabolism in pigs during HS demands further investigation for a better understanding of the mechanism, especially with regard to the effect of duration of HS on lipid mobilization.

Heat stress increases HSP70 mRNA abundance in both muscle and liver in boars and a tendency for HSP70 induction at the protein level in the mesenteric fat in boars and gilts. Therefore, there appears to be tissue- and sex-specific differences in the regulation of HSP70 during HS in pigs. This result also suggests that there may discordance in the level of HSP70 at the protein and mRNA levels or that this may suggest the existence of a dynamic regulation of HSP70 protein and mRNA abundance during chronic HS periods, leading to differences in the abundance of the protein and mRNA for HSP70 at a given time. As a chaperone, HSP70 helps protein refolding and prevents protein aggregation under HS. It also has anti-inflammatory properties through which it may suppress inflammatory cytokine release or action. The lack of an increase in

inflammatory cytokine (IL-6 and TNF-α) expression in pigs under HS may be related to the role of HSP70 in suppressing inflammation and is consistent with previous findings of attenuated inflammation in pigs under HS (Noble et al., 2008; Lambert, 2009; Geiger and Gupte, 2011; Welc et al., 2013).

Induction of CHOP protein in the mesenteric fat of HS pigs may suggest the presence of endoplasmic reticulum (ER) stress in HS pigs. It previously has been established that HS results in increased unfolded protein accumulation within the cell, which accumulates in the ER, inducing ER stress and unfolded protein response (Xu et al., 2011). CHOP is a critical marker for survival under HS (Minamino et al., 2010). Therefore, cellular proteins in pigs under HS may be protected by both HSP70 (cytoplasm) and CHOP (ER) induction. Mild ER stress occurs during adipocyte differentiation (Basseri et al., 2009), and it is possible that ER stress may play some role in the observed increase in lipid storage in adipocytes under HS.

The mechanism of perturbation of lipid metabolism during HS in pigs is intriguing. The upregulation of LPL mRNA abundance in the subcutaneous fat of HS pigs is in accordance with the increase in LPL activity in the subcutaneous fat of pigs under HS (Christon, 1988; Kouba, 2001). Increased LPL mRNA abundance and activity will result in increased influx of FFA into the cell. Increased concentration of intracellular FFA is cytotoxic and FFA is either rapidly oxidized or esterified within the cell (Havel et al., 1962). Fatty acid storage in neutral lipids in TAG or even phospholipids requires a molecule of glycerol for every molecule of TAG. Although glycerol can be synthesized from glucose during glycolysis, glyceroneogenesis is the major pathway for triglyceride glycerol in adipocytes (Nye et al., 2008). This is even more important because HS is a state of reduced glucose availability when glycolytic flux is reduced (Hall et al., 1980). In support of a major role for glyceroneogenesis, HS leads to upregulation of PCK1 abundance in the mesenteric and subcutaneous adipose depot fat compared with the PF and CON treatments. Although both GK and PCK1 are important for glyceroneogenesis, PCK1 is the dominant glyceroneogenic enzyme in adipose tissue (Hanson et al., 1970; Reshef et al., 1970) whereas GK activity is minimal (Robinson and Newsholme, 1967; Newsholme and Taylor,

1969). The induction of PCK1 in adipose tissue of HS animals is in agreement with our in vitro data showing elevated PCK1 expression in pig adipocytes differentiated under HS (Qu et al., 2015). Although PCK1 regulates gluconeogenesis in the liver, HS did not regulate its expression in the liver, and this may further explain the lack of treatment differences on serum glucose concentration in this study. Therefore, PCK1 induction appears specific to adipose tissue response under HS. This underscores the importance of glyceroneogenesis for the increased lipid storage during HS. It is intriguing that increased glyceroneogenesis and lipid storage in adipose tissue could occur during a period of reduced FI. Adaptive responses during HS are geared toward maximizing cellular survival. Therefore, it is conceivable that increased lipid storage during HS confers cellular survival advantage. In support of this possibility, glycerol is a known chemical chaperone and osmolyte (Kim et al., 2006) that may help meet the increased need for protein refolding during HS. Additionally, HSP70 translocates to the surface of lipid droplets (Jiang et al., 2007), where it may be involved in the protection of critical cellular proteins at the lipid droplet surface. The role of lipid droplet–associated HSP is a subject of intense interest among scientists (Welte, 2007).

In summary, we have provided evidence of tissue- specific differences in response to HS in pigs. Notably, we have shown that chronic HS did not involve major changes in glucose and insulin concentrations in the blood, despite the significant reduction in FI. In addition, serum concentrations of FFA and TAG were unaltered, perhaps reflective of metabolic adaptation during HS that led to preservation of lipids in adipose tissue. In support of this, HS led to induction of PCK1 in adipose tissue, indicating that glyceroneogenesis, regulated by PCK1, may be very important in the increased storage of lipids in adipocytes and adipose tissue of pigs under HS.

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Table 3.1 Feed ingredient composition (as-fed basis).

Item	Percentage
Ingredient	
Corn	79.75
Soybean meal	1.95
Dried distiller's grains	15.00
Swine white grease	1.00
L-lysine hydrochloride	0.38
L-threonine[1]	0.09
L-tryptophan	0.04
Limestone	1.14
Monocalcium phosphate	0.14
Vitamin premix[2]	0.10
Sodium chloride	0.25
Selenium 270 premix[3]	0.03
Phytase[4]	0.08
Non-sulfur T.M. premix[5]	0.05
Energy and nutrient	
CP, %	12
ME, Mcal/kg	3.37

[1] 98% L-threonine. [2]Swine vitamin concentration in premix: vitamin A, 544,680 IU/kg; vitamin D_3, 54,448 IU/kg; vitamin E, 3631 IU/kg; menadione (vitamin K), 182 mg/kg; vitamin B_{12}, 3.2 mg/kg; riboflavin, 726 mg/kg; d-pantothenic acid, 1816 mg/kg; niacin, 2723 mg/kg, biotin, 18.1 mg/kg; folic acid, 136 mg/kg; choline, 45,390 mg/kg; pyridoxine, 409 mg/kg; vitamin E, 1816 IU/kg; chromium, 16.3 mg/kg; carnitine, 4805 mg/kg. [3]Selenium 270 premix: 600 mg/kg of selenium as sodium selenite in a mixture of equal portions of ground limestone and ground corn to fill 0.05% of the diet. [4]600000 μg/kg phytase. [5]Non-sulfur trace mineral premix: iron, 51.05%; zinc, 20.73%; manganese, 2.86%; copper, 1.56%; iodine, 0.046%.

Table 3.2 Primer sequences for real time-PCR.

Gene[1]	Forward	Reverse
18S	5'-ATC CCT GAG AAG TTC CAG CA-3'	5'-CCT CTT GGT GAG GTC GAT GT-3'
aP2	5'-TGG TAC AGG TGC AGA AGT GG -3'	5'-ATT CTG GTA GCC GTG ACA CC -3'
Adiponectin	5'-TTT CTG GGC CCA CTG TGT TT-3'	5'-GGT TTT GCA TTG CAG GCT CA-3'
CD36	5'-ATC GTG CCT ATC CTC TGG-3'	5'-CCA GGC CAA GGA GGT TAA-3'
DGAT2	5'-CAC CTA CTC CTT CGG GGA GA-3'	5'-CTT GGA GTA GGG CAT GAG CC-3'
FAS	5'-AGT TTG TGA TGG AGA ACA CGG CCT -3'	5'-TGT TCA CAC GTG GTG CAA GGG TTA -3'
FATP4	5'-CAT TGT GGC TCA GCA GGT TA-3'	5'-CAG GCT AGG GGT CAA ATC AA-3'
FATP6	5'-TTC TTC GGCTAT GCT GGC AA-3'	5'-TGG ACC ATTAGG TCT CCG GT-3'
GK	5'-CGC TGA GGA AAG TGA AAT CCG -3'	5'-TCG CGT CTT TGG AAT CTA CGA -3'
GLUT4	5'-GAA GGA AGA AGG CAA TGC TG-3'	5'-GAG GAA CCG TCC AAG AAT GA-3'
HSP70	5'-TTC GTG GAC AGA AGC CAC AG -3'	5'-TTG CTA GGA TCT CCA CCC GA -3'
IL-6	5'-TCT GGG TTC AAT CAG GAG ACC TGC-3'	5'-TGC ACG GCC TCG ACA TTT CCC-3'
Leptin	5'-TTG GCC CTA TCT GTC CTA CG-3'	5'-GTG ACC CTC TGT TTG GAG GA-3'
LPL	5'-ATT CAC CAG AGG GTC ACC TG-3'	5'-AGC CCT TTC TCA AAG GCT TC-3'
PCK1	5'-CCC TGC CTT TGA AAA AGC CC -3'	5'-GGA GAT GAT TTC TCG GCG GT -3'
TNF-α	5'-CGT CGC CCA CGT TGT AGC CAA T-3'	5'-GCC CAT CTG TCG GCA CCA CC-3'

[1]aP2: fatty acid binding protein 2; CD36: fatty acid translocase 36; DGAT2: diglyceride acyltransferase 2; FAS: fatty acid synthase; FATP4: fatty acid transport protein 4; FATP6: fatty acid transport protein 6; GK: glycerol kinase; GLUT4: glucose transporter protein type 4; HSP 70: heat shock protein 70; IL-6: interleukin 6; LPL: lipoprotein lipase; PCK1: phosphoenolpyruvate carboxykinase 1; TNF-α: tumor necrosis factors α.

Table 3.3 Effects of heat stress on performance in pigs.

	Treatment			Sex			*P*-value	
Variable[1]	CON	PF	HS	Boar	Gilt	SEM	Trt	Sex
IBW, kg	74.48	74.13	74.3	66.80[a]	81.80[b]	1.77	0.720	<0.01
FBW, kg	76.20	73.49	75.35	67.59[a]	82.43[b]	1.68	0.902	<0.01
ΔBW, kg	1.72	-0.64	1.05	0.78	0.63	0.50	0.181	0.718
IRT, °C	38.9	38.9	39.0	38.87	38.93	0.05	0.723	0.863
FRT, °C	38.6[a]	38.3[a]	38.9[b]	38.63	38.53	0.07	<0.01	0.491
ΔRT, °C	-0.3[ab]	-0.6[a]	-0.05[b]	-0.23	-0.40	0.07	0.008	0.440

[1]IBW: initial body weight; FBW: final body weight; ΔBW: body weight change; IRT: initial rectal temperature; FRT: final rectal temperature; ΔRT: rectal temperature change; RR: respiration rate. [2]If the interaction effect is not significant, remove this effect in the model. If significant, use the original model. Different subscripts represent differences at $P < 0.05$.

Table 3.4 Effects of heat stress on subcutaneous fat mRNA level in pigs.

	Treatment			Sex			*P*-value	
Variable[1]	CON	PF	HS	Boar	Gilt	SEM	Trt	Sex
HSP70	1.07	0.95	1.43	0.94[a]	1.35[b]	0.13	0.459	0.044
PCK1	0.34[a]	0.69[a]	7.54[b]	4.31[b]	1.40[a]	1.00	<0.01	0.023
FAS	1.32	1.05	1.17	0.96	1.39	0.11	0.700	0.055
LPL	1.27[a]	0.81[b]	1.40[a]	1.19	1.13	0.09	0.004	0.809
Adiponectin	1.10	1.06	1.48	1.07	1.35	0.14	0.572	0.338
Leptin	2.48[ab]	1.53[a]	3.78[b]	3.22	1.97	0.38	0.030	0.134
aP2	1.09	0.96	1.31	0.92[a]	1.32[b]	0.10	0.229	0.020
CD36	1.06	0.93	1.38	1.16	1.07	0.10	0.214	0.792
FATP4	1.04	1.33	1.24	1.30	1.10	0.13	0.581	0.894
FATP6	1.21	1.01	1.10	1.17	1.05	0.07	0.557	0.573
GLUT4	1.70[b]	0.76[a]	1.46[ab]	1.61[b]	1.00[a]	0.15	0.016	0.006
DGAT2	1.82	0.93	1.32	1.64[b]	1.07[a]	0.16	0.094	0.026
IL-6	1.50	0.92	1.64	1.75[b]	0.95[a]	0.20	0.562	0.010
TNF-α	1.31	1.11	1.03	0.82[a]	1.47[b]	0.13	0.811	0.012

[1]aP2: fatty acid binding protein 2; CD36: fatty acid translocase 36; DGAT2: diglyceride acyltransferase 2; FAS: fatty acid synthase; FATP4: fatty acid transport protein 4; FATP6: fatty acid transport protein 6; GLUT4: glucose transporter protein type 4; HSP 70: heat shock protein 70; LPL: lipoprotein lipase; PCK1: phosphoenolpyruvate carboxykinase 1; TNF-α: tumor necrosis factors α. Different subscripts represent differences at $P < 0.05$

Table 3.5 Effects of heat stress on mesenteric fat mRNA level in pigs.

	Treatment			Sex			*P*-value	
Variable[1]	CON	PF	HS	Boar	Gilt	SEM	Trt	Sex
HSP70	1.17	1.17	1.64	1.15	1.50	0.15	0.334	0.145
PCK1	0.63[a]	1.70[ab]	6.79[b]	4.55[b]	1.52[a]	0.91	<0.01	0.025
FAS	1.00	1.57	1.01	0.75[a]	1.64[b]	0.16	0.345	<0.01
LPL	0.96	1.14	1.38	1.36[b]	0.96[a]	0.12	0.417	0.044
Adiponectin	1.16	1.16	1.32	1.58[b]	0.85[a]	0.17	0.845	0.007
Leptin	0.73	0.76	2.66	1.78	0.98	0.35	0.174	0.500
aP2	1.05	1.31	1.14	1.17	1.16	0.09	0.540	0.725
CD36	0.83	1.33	1.21	1.15	1.09	0.10	0.121	0.545
FATP4	0.98	1.74	2.29	0.79[a]	2.54[b]	0.35	0.437	<0.01
FATP6[2]	1.29	1.31	1.42	1.92	0.76	0.15	0.829	<0.01
GLUT4	1.60	2.01	1.99	2.97[b]	0.91[a]	0.33	0.539	<0.01
DGAT2	1.20	1.05	1.75	1.65[b]	1.01[a]	0.17	0.432	0.013
IL-6	1.55	1.51	0.89	1.35	1.28	0.21	0.708	0.896
TNF-α	0.99	1.75	1.11	1.02	1.55	0.19	0.510	0.177

[1]aP2: fatty acid binding protein 2; CD36: fatty acid translocase 36; DGAT2: diglyceride acyltransferase 2; FAS: fatty acid synthase; FATP4: fatty acid transport protein 4; FATP6: fatty acid transport protein 6; GLUT4: glucose transporter protein type 4; HSP 70: heat shock protein 70; LPL: lipoprotein lipase; PCK1: phosphoenolpyruvate carboxykinase 1; TNF-α: tumor necrosis factors α. Different subscripts represent differences at $P < 0.05$. [2] $P < 0.01$ for FATP6 interaction.

Table 3.6 Effects of heat stress on mRNA level of selected genes in the liver of pigs.

	Treatment			Sex			*P*-value	
Variable[1]	CON	PF	HS	Boar	Gilt	SEM	Trt	Sex
PCK1	2.15	2.14	1.44	1.40	2.42	0.42	0.227	0.062
GK	1.09	1.66	1.10	1.03	1.53	0.16	0.322	0.134
FAS	1.94	1.31	0.85	1.89[b]	0.83[a]	0.21	0.061	<0.01

[1]PCK1: phosphoenolpyruvate carboxykinase 1; GK: glycerol kinase; FAS: fatty acid synthase. Different subscripts represent differences at $P < 0.05$.

Table 3.7 Effects of heat stress on mRNA level of selected genes in the LD muscle of pigs[1].

	Treatment			Sex			*P*-value	
Variable[2]	CON	PF	HS	Boar	Gilt	SEM	Trt	Sex
PCK1	1.25	1.11	4.90	2.98	1.16	0.48	0.507	0.178
GK	1.19	1.36	1.20	1.40	1.10	0.16	0.803	0.900

[1]LD: longissimus dorsi muscle. [2]PCK1: phosphoenolpyruvate carboxykinase 1; GK: glycerol kinase; Different subscripts represent differences at $P < 0.05$.

Figure 3.1 Heat stress (HS) effects on daily feed intake, skin temperature and respiration rate.

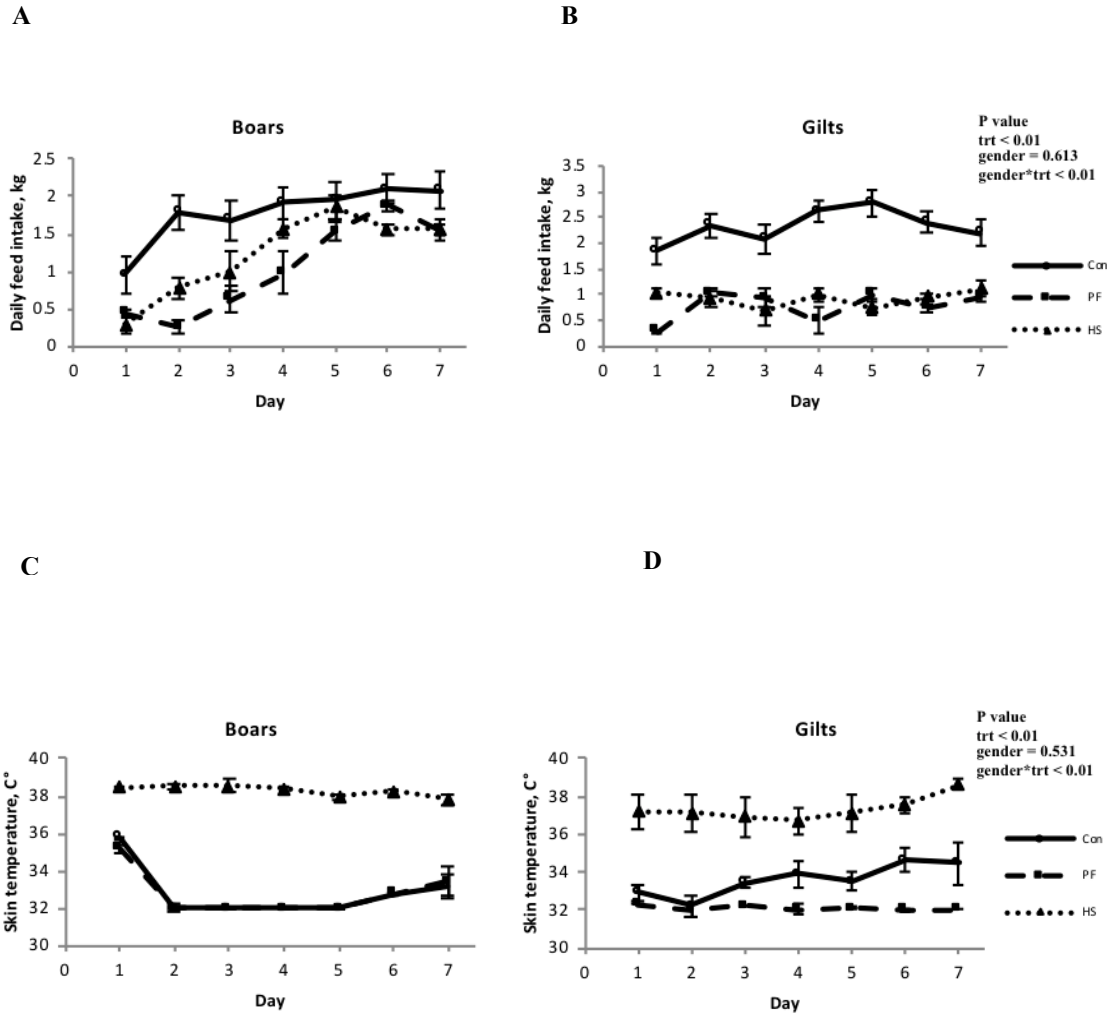
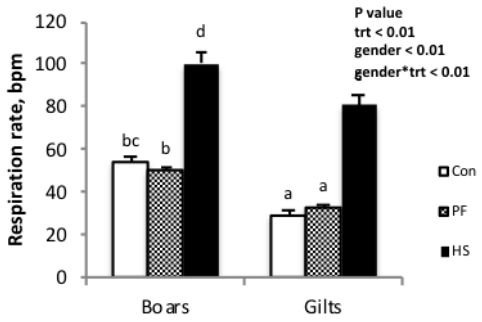


Figure 3.1 Continued

E



Control (CON, 20 °C with ad libitum feed intake), pair-fed (PF, 20°C with pair feeding to heat stress treatment), and heat stress (35°C with ad libitum feed intake).(A) Boars feed intake. (B) Gilts feed intake. (C) Boars skin temperature. (D) Gilts skin temperature. (E) Respiration rate. Bars represents means ± SEM. $^{a\text{-}d}P<0.05$.

Figure 3.2 Heat stress (HS) effects on plasma metabolites.

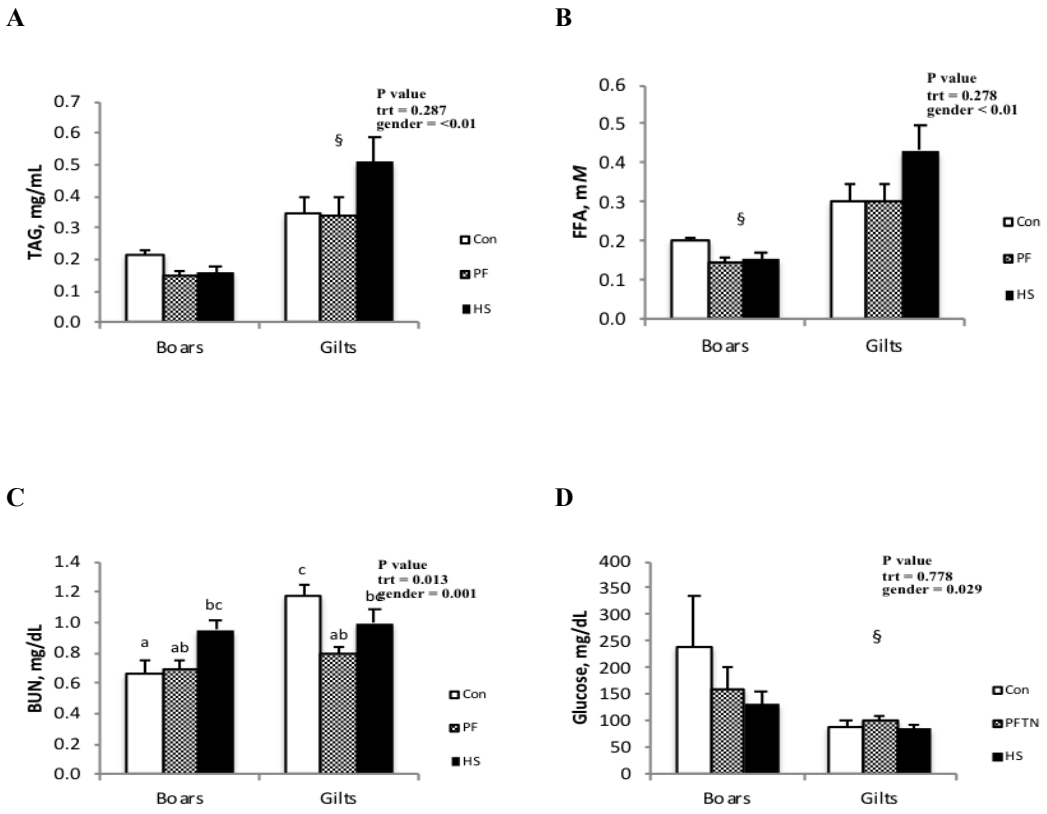
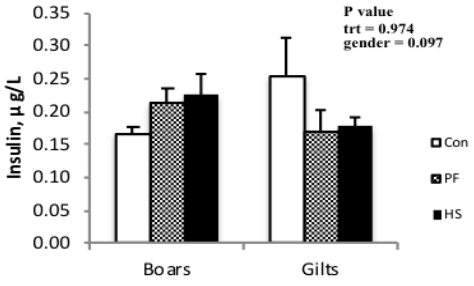
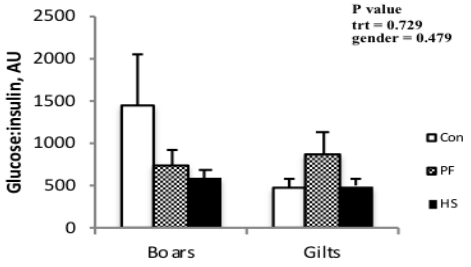


Figure 3.2 Continued

E

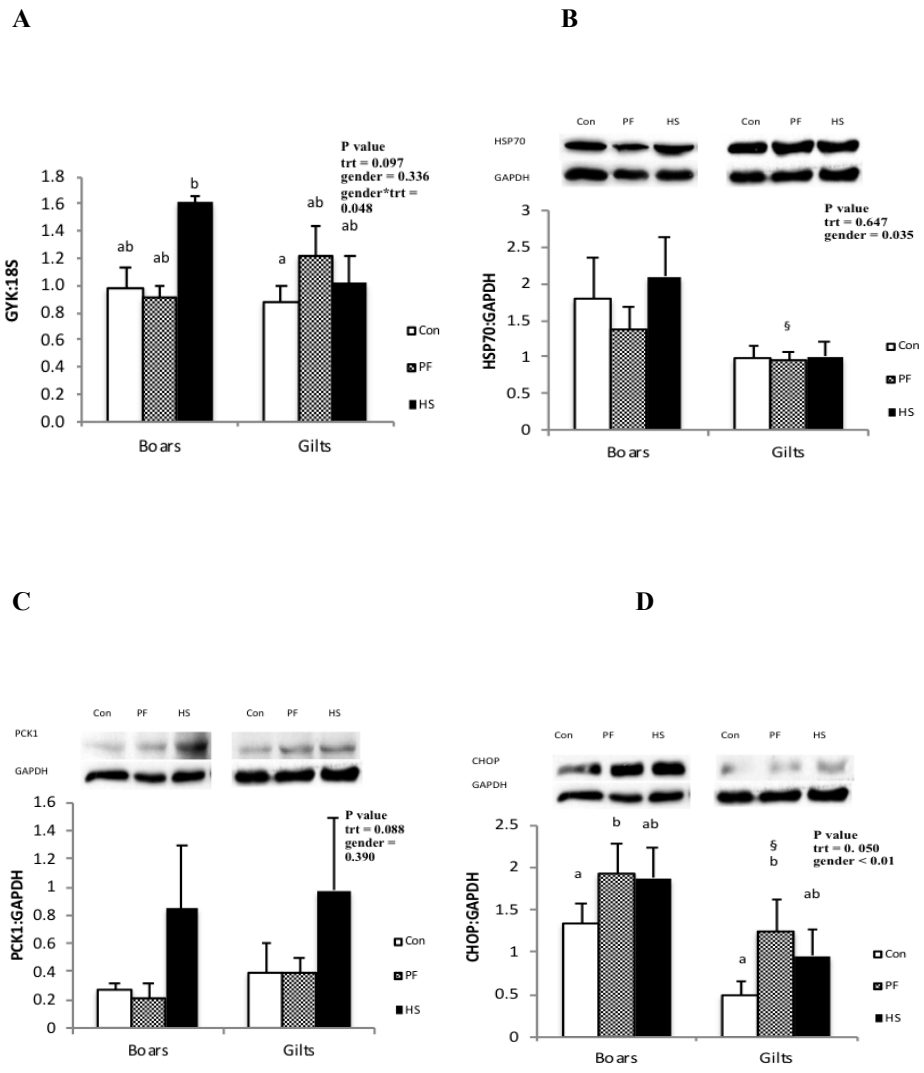


F



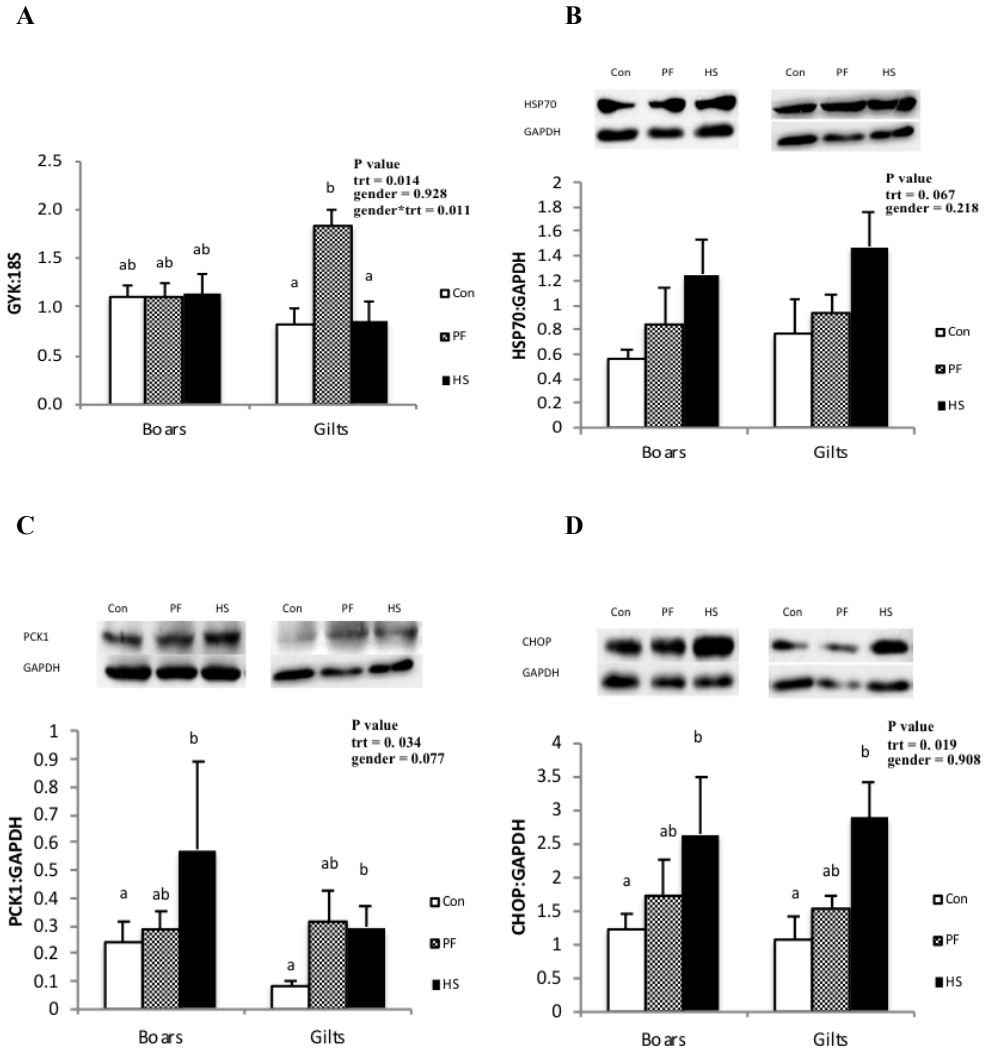
Control (Con, 20 °C with ad libitum feed intake), pair-fed (PF, 20°C with pair feeding to heat stress treatment), and heat stress (35°C with ad libitum feed intake) environments. (A) Triglyceride (TAG). (B) Free fatty acid (FFA). (C) Blood urea nitrogen (BUN). (D) Glucose. (E) Insulin. (F) Glucose to insulin ratio. Bars represents means ± SEM. $^{a\text{-}d}P<0.05$. § means significant sex effect.

Figure 3.3 Heat stress (HS) effects on gene and protein levels of subcutaneous fat.



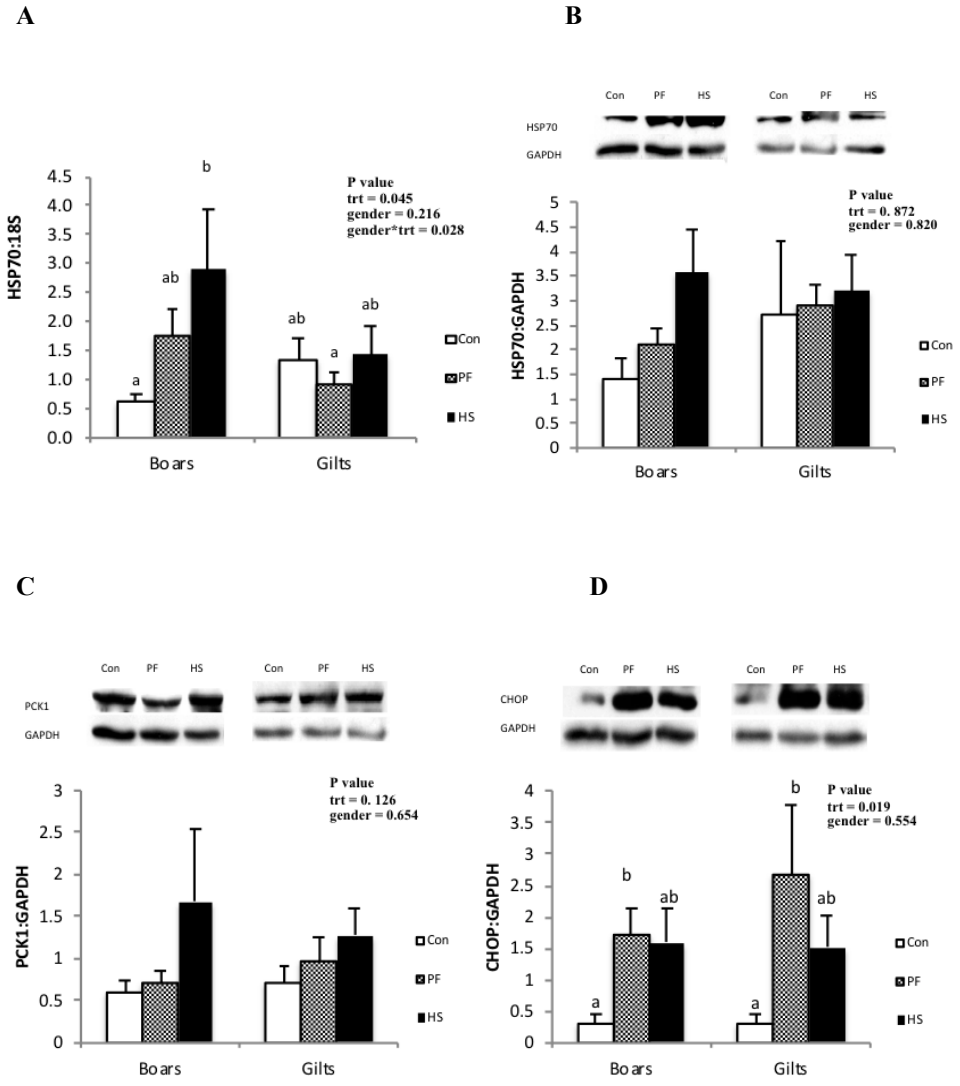
Control (CON, 20 °C with ad libitum feed intake), pair-fed (PF, 20°C with pair feeding to heat stress treatment), and heat stress (35°C with ad libitum feed intake) environment. (A) Glycerol kinase (GK) mRNA level. (B) Heat shock protein 70 (HSP70) protein level. (C) Phosphoenolpyruvate carboxykinase (PCK1) protein vel. (D) CCAAT-enhancer-binding protein homologous protein (CHOP) protein level. Bars represents means ± SEM. $^{a\text{-}d}P<0.05$. § means significant sex effect.

Figure 3.4 Heat stress (HS) effects on gene and protein level of mesenteric fat.



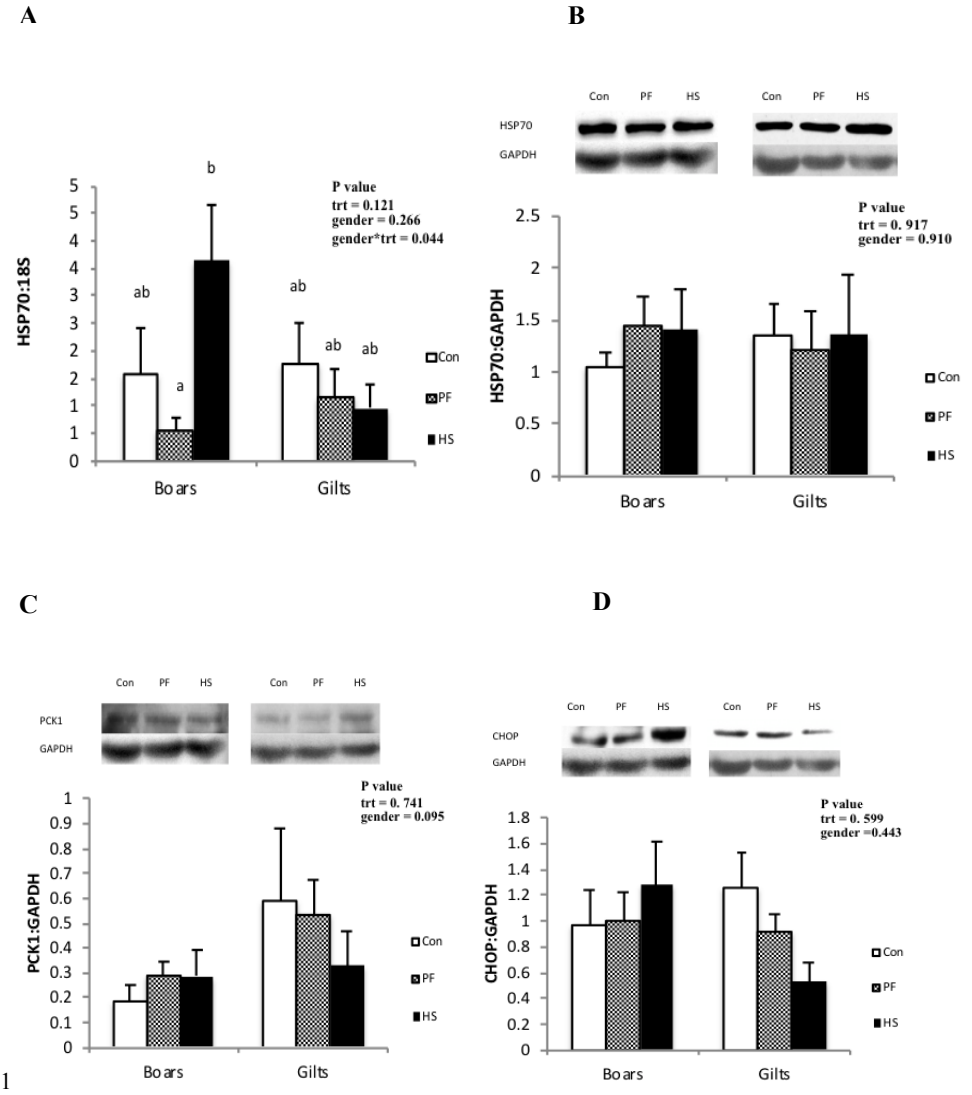
Control (CON, 20 °C with ad libitum feed intake), pair-fed (PF, 20°C with pair feeding to heat stress treatment), and heat stress (35°C with ad libitum feed intake) environment. (A) Glycerol kinase (GK) mRNA level. (B) Heat shock protein 70 (HSP70) protein level. (C) Phosphoenolpyruvate carboxykinase (PCK1) protein level. (D) CCAAT-enhancer-binding protein homologous protein (CHOP) protein level. Bars represents means ± SEM. $^{a\text{-}d}P<0.05$. § means significant sex effect.

Figure 3.5 Heat stress (HS) effects on gene and protein levels in liver.



Control (CON, 20 °C with ad libitum feed intake), pair-fed (PF, 20°C with pair feeding to heat stress treatment), and heat stress (35°C with ad libitum feed intake) environments. (A) Heat shock protein 70 (HSP70) mRNA level. (B) Protein level of HSP70. (C) Phosphoenolpyruvate carboxykinase (PCK1) protein level. (D) CCAAT-enhancer-binding protein homologous protein (CHOP) protein level. Bars represents means ± SEM. $^{a\text{-}d}P<0.05$. § means significant sex effect.

Figure 3.6 Heat stress (HS) effects on gene and protein levels in longissimus dorsi muscle.



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Control (CON, 20 °C with ad libitum feed intake), pair-fed (PF, 20°C with pair feeding to heat stress treatment), and heat stress (35°C with ad libitum feed intake). (A) Heat shock protein 70 (HSP70) mRNA level. (B) Protein level of HSP70. (C) Phosphoenolpyruvate carboxykinase (PCK1) protein level. (D) CCAAT-enhancer-binding protein homologous protein (CHOP) protein level. Bars represents means ± SEM. $^{a\text{-}d}P<0.05$. § means significant sex effect.

CHAPTER 4. ADIPOSE TISSUE SPECIFIC RESPONSES REVEAL AN IMPORANT ROLE OF LIPOGENESIS DURING HEAT STRESS ADAPATATION IN PIGS

4.1 Abstract

Elevated ambient temperature causes heat stress in pigs, resulting in reduced animal performance. To better understand tissue responses to heat stress in pigs, we conducted a study in which pigs were subjected to four treatments: acute (24 h) heat stress (AHS) at 35°C ± 1 ambient temperature, chronic (7 d) heat stress at 35°C ± 1 (HS) or normal ambient temperature (20°C± 1) for 7 d with ad-libitum feeding (Con) or with pair-feeding to the feed intake (FI) of the HS pigs (PF). Heat stress decreased FI by approx. 36% after 1 wk of treatment in HS treatments and 64% after 1d of treatment in AHS treatments, compared with Con ($P < 0.01$). Concentration of free fatty acids (FFA) was elevated in AHS compared to HS ($P = 0.031$). Serum insulin concentration was lower in PF than Con ($P = 0.045$). Blood urea nitrogen (BUN) concentration was elevated in HS compared with Con and PF ($P = 0.008$), but lower ($P < 0.021$) in AHS compared to HS. In the subcutaneous adipose tissue, the mRNA and protein abundance of PCK1 were higher ($P < 0.05$) in the HS treatment than Con and PF, and also higher ($P < 0.05$) in HS than AHS. However, there was no difference in glycerol kinase (GK) mRNA between Con, PF and HS, although its mRNA level was lower ($P = 0.003$) in AHS vs. HS. Protein abundance of the endoplasmic reticulum (ER) stress marker, CCAT box/enhancer-binding homologous protein (CHOP), was higher in PF than Con ($P < 0.05$), and higher ($P = 0.033$) in HS than AHS in subcutaneous fat. In mesenteric fat, PCK1 mRNA level was higher ($P < 0.001$) in the HS than Con and PF treatments. Additionally, mRNA level of PCK1 was lower ($P = 0.039$) in AHS vs. HS. mRNA level of PCK1 was downregulated ($P < 0.05$) in the liver of PF pigs compared to other treatments, but most other genes measured were not affected by treatment in the liver and muscle tissues. These results confirm that heat stress induces a robust adipose tissue response in favor of increased lipid storage. This indicates that adipose tissue might play an important role in heat adaptation.

Key words: adipose tissue, CHOP, heat stress, PCK1 or PEPCK, pig

4.2 Introduction

Heat stress is one of the costliest problems in the swine industry in worldwide (St-Pierre et al., 2003). Heat stress increases morbidity and mortality and has a negative impact on performance and carcass quality in pigs (White et al., 2008). Heat stress also results in increased adipose tissue deposition through an upregulation of lipogenic enzymes such as lipoprotein lipase (Christon, 1988; Kouba et al., 2001). We have previously reported that, despite hyper-catabolic effects of heat stress, pig adipocytes show enhanced lipogenesis in a cell autonomous manner (Qu et al., 2015), suggesting a direct sensing of heat by adipocytes. Using the wild Ossabaw wild miniature pig model, we also demonstrated that heat stress led to an upregulation of protein and mRNA of glyceroneogenesis markers, especially phosphoenolpyruvate carboxykinase (PCK1 or PEPCK-C) and to a limited extent, glycerol kinase (GK), in subcutaneous adipose tissue (Qu et al., 2016). Although adipocytes express both GK (Guan et al., 2002; Qu et al., 2015) and PCK1 (Qu et al., 2015), the dominant glyceroneogenic enzyme in adipocytes is PCK1 (Reshef et al., 1970). This metabolic adaptation is expected to result in increased lipid storage due to the important role of PCK in the synthesis of glycerol, a precursor for triglyceride synthesis. Ossabaw pigs have a "thrifty" phenotype which allows them to store excess energy in adipose tissue (Lassaletta et al., 2012), and this may represent a survival mechanism. However, it remains to be confirmed whether increased lipid storage during heat stress represents a protective mechanism of heat stress adaptation, although glycerol is a known chemical chaperone that could help in preventing protein misfolding during heat stress (Engin and Hotamisligil, 2010). However, differences in adipose tissue responses during acute and chronic heat exposure are yet to be determined.

In this experiment, we have used a commercial-type pig genotype, a Duroc X Yorkshire X Landrace terminal cross, to evaluate effects of both acute (24 h) and chronic (7 d) heat stress on mRNA level responses in adipose and other peripheral tissues. Our objective was to determine tissue specific response to heat stress, especially metabolic responses that might increase our understanding of adaptive response to heat stress in pigs. We hypothesize that glyceroneogenesis

represents a major metabolic response during chronic exposure to temperature above thermoneutral in pigs.

4.3 Materials and methods

Animals and Experimental Design

The protocols and all animal care procedures described in these experiments were approved by the Purdue Animal Care and Use Committee. A total of 40 crossbred barrows, with mean initial body weight (IBW) of 72.68 ± 2 (SE) kg, were randomly allocated (10/treatment) to four treatments: control (Con) treatment with ad libitum feeding, with room temperature set at 20 °C ± 1(SE) °C and 53-78% relative humidity (RH) for 7 d; HS treatment with room temperature set at 35 °C ± 1(SE) °C, RH at 45-65% with ad libitum feed intake for 7 d (chronic exposure); pair-fed (PF) group with same room temperature and RH as the Con treatment, and pair-fed to the feed intake of heat HS group for 7 d; acute heat stress (AHS) treatment with room temperature at 35 °C ± 1 (SE)°C and RH at 45-65% with ad libitum feed intake for 24 h (acute exposure). Room conditions were observed every 3 h to ensure they remain within set limits. Pair-fed pigs were fed twice daily at 0900 and 0500 h with an amount of feed that was equal to the previous day's FI in the HS treatment. All pigs were fed a common diet that met or exceeded NRC (2012) requirement for pigs of similar size (Table 1). All pigs had free access to water. Animals were individually penned and allowed to adjust to their environment for 7 d before heat stress was applied to the HS and the AHS groups.

Body weights (BW) and rectal temperature (RT) were recorded at the start and the end of the experiment. Feed intake (FI) and skin temperature were measured daily during the experiment. Skin temperatures were measured using an infrared noncontact digital thermometer (model WIC-276664; Walgreens, Deerfield, IL). Rectal temperatures were measured with a digital rectal thermometer (model KD-113; Walgreens). Respiration rate (RR) was measured three times at the end of the experiment (d 1 for AHS and d 7 for Con, HS and PF) by visually observing and counting the rise and fall of the flank in a timed minute. About 10 ml of blood was collected into heparinized vacutainer tubes on d 0, d 1 (AHS pigs only). And d 7 of the experiment through

jugular venipuncture. Plasma was separated from whole blood through centrifugation at 10,000 × g for 10 min at 4 ºC. Pigs were euthanized after sedation with intramuscular injection of atropine, tiletamine-zolazepam and xylazine, and CO_2 asphyxiation, followed by pneumothorax and cardiectomy while anesthetized. After sacrifice, adipose tissues, subcutaneous adipose tissue (collected from the middle layer at the 10th rib) and mesenteric adipose tissue (from the ileum), longissimus dorsi (LD) and semitendinosus (STD) muscles and liver were collected on d1 (AHS) or d 7 (Con, PF and HS treatments) of experiment. Tissues were snap frozen in liquid nitrogen immediately after collection and stored in -80 ºC before RNA and protein extraction.

mRNA level *Analysis*

mRNA level of metabolic and inflammatory genes in subcutaneous adipose tissue, liver, and muscle was determined by real-time PCR. QIAzol lysis reagent (Qiagen, Valencia, CA) was used for RNA extraction using approximately 0.5 g of tissue. RNA was dissolved in nuclease-free water (Ambion, Austin, TX). Concentrations of RNA were measured with a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Integrity of RNA and genomic DNA contamination was checked by electrophoresis on 0.8% agarose gel. RNA was reverse transcribed with the Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, WI). PCR assay was conducted on a Bio-Rad MyiQ thermocycler (Bio-Rad, Temecula, CA) with the RT2 SYBR green qPCR mastermix (Qiagen, Valencia, CA) in a total reaction volume of 20 μL. Sequences of gene-specific PCR primers are presented in Table 2. Level of mRNA of each gene was calculated after its cycle threshold (Ct) was normalized to the Ct for 18S using the ΔΔCt method.

Western Blot Analysis

Tissues were homogenized in 1X radio-immunoprecipitation assay (RIPA) buffer [50 M Tris-HCl (Ameresco, Solon, OH), 0.25% Deoxycholic acid (Sigma-Aldrich, St. Louis, MO), 15 M NaCl, and 10 mM EDTA (Sigma-Aldrich, St. Louis, MO), 0.1% Triton X (Sigma-Aldrich, St. Louis, MO)] supplemented with commercial protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO). Homogenates were centrifuged at 10,000 × g for 10 min at 4 °C. Protein concentration was measured with the bicinchoninic acid assay (Thermo Fisher

Scientific, St. Louis, MO). For each sample, equal amount of protein (40 μg) was resolved on 10% SDS polyacrylamide gels. Proteins were transferred from the gel to nitrocellulose membranes (Bio-Rad Laboratories, Temecula, CA). Membranes were blotted with primary antibodies: anti-β-actin (Cell Signaling Technology, Danvers, MA), anti-heat shock protein (HSP) 70 (Cayman Chemicals, Ann Arbor, MI), anti-PCK1 (Abcam Inc., Cambridge, MA), anti-CCAT/enhancer-binding homologous protein (CHOP, Cell Signaling Technology, Danvers, MA). Secondary antibodies were either horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit IgG (Cell Signaling, Danvers, MA). Chemiluminescent signals were developed with Immobilon® HRP substrate (Millipore, Billerica, MA) and captured by the autoradiographic film exposure (Santa Cruz Biotechnology, Dallas, TX). Band intensities of specific proteins and the housekeeper (β-actin) were determined with a Kodak 1 D 3.6 software (Eastman Kodak, Rochester, NY). For each sample, band intensity of each detected protein was normalized to that of β-actin.

Plasma Metabolite Measurements

Total plasma triglyceride (TAG) content was measured using a TAG determination kit (Sigma-Aldrich, St Louis, MO). Plasma free fatty acid (FFA) was determined using a non-esterified fatty acid kit (NEFA) kit with intra- and inter-assay CVs of 0.8% and 3.7% (Wako Diagnostics, Richmond, VA). Blood urea nitrogen (BUN) was measured with BUN colorimetric detection kit with intra-assay CV of 4.3% (Arbor Assays, Ann Arbor, MI). Plasma glucose was measured with a commercially available kit (Wako Diagnostics, Richmond, VA). The sensitivity of this assay was 0.7 mg/dl, and the intra- and inter-assay CV were both less than 5%. Plasma insulin was determined using the Mercodia porcine insulin ELISA kit with intra-assay CV of 4.7% (Mercodia, Uppsala, Sweden).

Statistical Analyses

Data were analyzed to determine differences between 7-day treatments (CON, PF and HS) by one-way ANOVA with treatment and animal considered fixed and random effects. This was followed by Tukey's mean separation test (SAS Inst. Inc., Cary, NC). Data between high temperature treatments (HS and AHS) were analyzed by one-way ANOVA with treatment and

animal considered fixed and random effects. This was followed by paired t-test. Box-Cox transformation procedure was applied when residuals were not normal. Results were considered significant with $P < 0.05$ and were regarded as showing a strong tendency of significance with P-values between 0.05 and 0.10. Data are presented as means ± SEM.

4.4 Results

Animal Performance

Feed intake of the PF, HS and the AHS treatments were lower ($P < 0.05$) than Con in the first 24 h of the study (Fig. 4.1A). After 7 d, FI was decreased by approximately 36% in HS treatment compared with Con ($P < 0.05$; Fig. 4.1B). Body weight (BW) change (ΔBW) was positive and similar in PF and HS pigs, but less than in the Con pigs ($P < 0.01$; Table 4.3). The HS treatment gained a total of 1.8 ± 0.2 kg, whereas the AHS treatment resulted in weight loss of approx. -1.4 ± 0.1 kg. Skin temperature was higher ($P < 0.001$) by approx. 2°C in HS and AHS compared with Con and PF in the first 24 h of the study (Fig. 4.1C). This difference remained between HS, Con and PF after 7 d of the study (Fig. 1D). There was greater and positive rectal temperature change in HS vs. Con. ($P < 0.043$) (Table 4.3). However, there was no difference in this change between HS and AHS ($P = 0.731$). Respiration rate was 2-fold higher ($P = 0.023$) in HS compared to Con and PF (Fig. 4.1E). The AHS treatment had significantly lower ($P < 0.037$) respiration rate compared with HS.

Plasma Metabolites

Plasma TAG was elevated in the AHS treatment compared with PF (1.7-fold, $P = 0.049$; Fig. 4.2A). There was also an elevated FFA in the AHS pigs compared to Con and PF pigs (2-fold, $P = 0.004$; Fig. 4.2B). However, there were no treatment effects on plasma glucose ($P =0.685$, Fig. 4.2C) and glucose to insulin ratio ($P =0.446$, Fig. 4.2E). Insulin concentration was lower in PF than Con ($P =0.045$, Fig. 4.2D). In addition, BUN concentration was elevated in both HS and AHS treatments compared with Con and PF ($P =0.023$, Fig. 4.2F). However, BUN was lower ($P = 0.021$) in AHS compared to HS.

Subcutaneous Fat Specific Responses to HS

There was an induction of HSP70 mRNA level in HS compared with Con and PF ($P = 0.003$, Table 4.4). mRNA level of HSP70 was similar between HS and AHS. At the protein level, a higher HSP70 protein was found in the HS treatment compared with Con ($P = 0.013$, Fig. 4.3A). However, HSP70 protein was lower in AHS compared to HS ($P = 0.009$). The mRNA and protein abundance of PCK1 were also higher in the HS treatment than Con and PF ($P < 0.001$, Table 4.4, and $P = 0.048$, Fig. 4.3B), and also higher in HS than AHS. However, there was no difference in GK mRNA between Con, PF and HS (Table 4.4), although its mRNA level was lower ($P = 0.003$) in AHS compared with HS. The mRNA level of AP2 (adipocyte Protein 2) was elevated in HS compared to PF (1.5-fold, $P = 0.035$, Table 4.4). There was no difference in the mRNA level of GLUT4 between Con, PF and HS ($P = 0.046$, Table 4.4), although its mRNA level was lower in AHS vs. HS ($P < 0.011$). Inflammatory cytokines were differentially regulated. mRNA level of IL-6 was higher in HS than Con and PF ($P < 0.001$, Table 4.4), and its mRNA level was also lower in AHS relative to HS ($P = 0.51$). However, the mRNA level of TNF-α was lower in HS and PF than Con ($P < 0.001$, Table 4.4), although there was no difference between HS and AHS ($P = 0.848$). Protein abundance of the ER stress marker, CHOP, was higher in PF than Con ($P = 0.019$), and also higher in HS than AHS ($P = 0.033$, Fig. 4.3C).

Mesenteric Fat Specific Responses to HS

There was no difference in HSP70 mRNA in treatments in mesenteric fat ($P = 0.361$, Table 4.5). Similarly, there was no treatment effect on the HSP70 protein level (Fig. 4.4A). Similar to findings in the subcutaneous adipose tissue, mRNA level of PCK1 was higher in the HS than Con and PF treatments ($P < 0.001$, Table 4.5). Additionally, mRNA level of PCK1 was lower ($P = 0.039$) in AHS vs. HS. However, there was no significant treatment difference in PCK1 protein level (Fig. 4.4B). mRNA level of LPL was lower ($P = 0.036$) in AHS compared with HS (Table 4.5). Leptin mRNA level was lower ($P = 0.012$) in PF than Con (Table 4.5). There was no treatment effect on adiponectin mRNA level. Furthermore, protein level of CHOP was not significantly affected by treatment (Fig. 4.4C).

Muscle and Liver Specific Responses to HS

mRNA level changes in the different treatments in muscle tissues are presented in Table 6. The mRNA abundance of HSP70 was elevated in HS compared with Con and PF in STD ($P < 0.001$) and LD muscles ($P = 0.048$) (Table 4.6). However, HSP70 mRNA level was lower ($P < 0.003$) in the LD of pigs in AHS compared to those in HS. There was a higher LPL mRNA level in STD muscle in HS than other treatments ($P < 0.001$, Table 4.6). mRNA level of LPL was lower in the STD of pigs in AHS compared to HS ($P < 0.001$). However, there was no treatment effect on LPL mRNA level in LD muscle (Table 4.6). mRNA level of FAS was lower in AHS compared to HS ($P = 0.034$). Pigs in the PF treatment had a higher liver PCK1 mRNA level than other treatments ($P = 0.047$, Table 4.7). Protein levels of HSP70, PCK1, and CHOP were not significantly affected by treatment in the LD and STD muscle and liver.

4.5 Discussion

Animals have robust mechanisms for adapting to both chronic and acute heat stress when in ambient temperature higher than their thermoneutral zone (Renaudeau et al., 2012). These adaptive mechanisms include behavioral and metabolic adaptations. Pigs will adapt to high temperature by ingesting copious amounts of water, increase recreational water use and reduce feed intake and activity (Kellner et al., 2016; Lucy and Safranski, 2017). At the cellular level, heat stress results in increased mRNA and protein level of chaperone proteins such as HSP70 that help prevent protein misfolding and in the renaturation of misfolded proteins (Brown, 1990; Clerico et al., 2015). Depending on the duration of excessive ambient temperature, heat stress may be acute or chronic (Pearce et al., 2015; Cui et al., 2016; Cruzen et al., 2017). However, differences in physiological responses such as changes in concentrations of metabolites, hormones and mRNA level of critical metabolic genes during different durations of heat stress exposure are still incompletely understood. Higher skin and rectal temperatures, and a higher respiration rate, were observed in HS and AHS compared to PF and Con. In addition, although acute heat stress led to weight loss in the AHS treatment, chronic heat stress in the HS group was associated with a modest positive weight gain, indicating heat adaptation in the HS group. This finding is consistent with previous findings that heat stress causes lower feed intake and reduced

body weight than when pigs are kept in a thermoneutral ambient temperature (Yunianto et al., 1997; Collin et al., 2001; Kouba et al., 2001; Pearce et al., 2013; Pearce et al., 2015).

Several tissue and metabolic changes observed in this experiment are consistent with our earlier observation in Ossabaw pigs (Qu et al., 2016) and in mice (Morera et al., 2012), that adipose tissue represents a major tissue location of heat stress response. One of the key metabolic changes was the increase in serum concentration of FFA in pigs in the AHS. Adipose tissue FFA is mobilized during periods of negative energy balance, and it plays an important role as an important substrate for energy generation in tissues through beta oxidation (Victoria Sanz Fernandez et al., 2015). It is quite interesting that the increased FFA concentration in AHS pigs had disappeared in HS pigs at the end of the 7d of chronic heat stress. This is similar to the pattern of FFA concentration reported in pigs exposed to acute (1 d) or chronic (7 d) heat stress (Pearce et al., 2013). This suggests a metabolic adaptation during chronic heat stress to prevent excessive mobilization of body adipose stores. This tendency for reduced serum FFA during chronic heat stress is supported by the elevated mRNA level of lipogenic markers including PCK1, GK, GLUT4, LPL, and aP2 in HS treatment compared with AHS in adipose tissue. Both GK and PCK1 are essential for glyceroneogenesis and PCK1 is recognized to play a dominant glyceroneogenic role in adipose tissue (Hanson et al., 1970; Reshef et al., 1970). Increased glyceroneogenesis through induction of PCK1 and GK in adipose tissue will enhance sequestration of FFA in triglycerides in adipose tissue, and this perhaps contributed to the reduced serum FFA concentration in HS treatment at d7. The elevation of PCK1 mRNA level in both the subcutaneous and mesenteric fat, and the absence of these tissue responses in AHS support the hypothesis that duration of heat stress is accompanied by different metabolic response in adipose tissue. The lack of treatment effect on serum TAG in this experiment is similar to our earlier observation in boars (Qu et al., 2016) and that of Pearce et al., (2013) which showed that heat stress did not alter TAG concentration, perhaps indicating that this metabolite is less subject to fluctuation during heat stress.

With pair-feeding, it was possible to separate observed effects in the HS group that might have been due to reduced feed intake. Despite the 50% reduction in caloric intake in the PF and HS groups relative to Con, distinct responses were observed in the HS group compared to PF,

confirming that these responses were independent of feed intake reduction. For example, in contrast to the induction of mRNA level of PCK1 in adipose tissue in the HS group, pair-feeding led to induction of PCK1 mRNA level in the liver of PF pigs. The elevation of PCK1 mRNA level in the liver could be a gluconeogenic response in the liver to provide additional glucose from this mechanism during this period of reduced nutrient intake. Thus, PCK1 may be insensitive to temperature regulation in the liver as in adipose tissue but may be more sensitive to energy balance status. The increased mRNA level of PCK1 in the liver of PF pigs may be related to the reduced insulin concentration in these pigs. Insulin is known to be a negative regulator of PCK1 mRNA level in the liver (Barthel and Schmoll, 2003). Therefore, the lowering of serum concentration of insulin would remove the negative effect of insulin on PCK1 mRNA level in the liver, allowing increased hepatic gluconeogenesis. However, the lack of difference in insulin concentrations between the HS and Con and HS and PF treatments indicates that responses in HS pigs were unrelated to alteration in circulating concentration. Leptin gene was not induced in adipose tissue by heat stress in the HS group in this study unlike in Ossabaw pigs that were exposed to a similar duration of chronic heat stress (Qu et al., 2016). Therefore, breed or genetic differences might exist in the regulation of leptin mRNA level in adipose tissue under heat stress.

Heat stress is known to cause increased muscle catabolism (Pearce et al., 2013; Cruzen et al., 2017; Ganesan et al., 2017). The protein catabolism indicator, BUN, was elevated by both HS and AHS treatments. Increased BUN concentration in heat stress has been previously reported (Pearce et al., 2013). Other tissue-specific response to heat stress include differential regulation of HSP70. Abundance HSP70 mRNA was induced in HS group in the subcutaneous fat, LD, and STD muscle, but not in the liver. Additionally, protein level of HSP70 was increased in subcutaneous adipose tissue in the HS, but not the AHS treatment. HSP70 functions as a chaperone protein and works in protein refolding to prevents protein denaturation (Verghese et al., 2012). The cytoprotective role of HSP70 also depends on its ability to inhibit apoptosis (Samali and Orrenius, 1998; Garrido et al., 2003). Therefore, these results may suggest that regulation of HSP70 mRNA level is tissue specific. Furthermore, HSP70 protein level was not induced in the AHS group compared to the HS, suggesting that duration of heat stress exposure may determine the induction of HSP70 at the protein level. This is in contrast to the induction of HSP70 protein in the LD muscle of pigs after 1d of heat stress (Pearce et al., 2013), perhaps

reflecting tissue specific differences or differences in experimental contexts. The reason for the difference in the regulation of LPL in the HS treatment in the STD and LD muscles are unclear but may be related to fiber type differences between these two muscle types. The STD muscle is known to have more red oxidative fibers than LD which has mostly white glycolytic fibers (Depreux, 2002). Substrate use between the two muscle fiber types are also different, with the red oxidative fibers using more fatty acids than the white fibers (Henriksson, 1990; Huber et al., 2007). Thus, the induction of LPL in STD during heat stress could allow intake of fatty acids for oxidation by the red fibers in this muscle.

Similarly, unlike its induction in subcutaneous adipose tissue in HS, CHOP protein level was not induced in AHS. CHOP is an endoplasmic reticulum (ER) stress marker. Heat stress results in increased unfolded protein response in which misfolded or unfolded proteins accumulate in the endoplasmic reticulum (ER), inducing ER stress (Fu et al., 2008; Xu et al., 2011). There was elevated protein level of CHOP in the subcutaneous adipose tissue of PF pigs compared to Con. This finding is similar to our previous finding in Ossabaw pigs (Qu et al., 2016). However, unlike our finding in Ossabaw pigs, CHOP was not induced by chronic heat stress in the HS group in mesenteric fat, suggesting that genetic differences may exist in the regulation of tissue protein level of CHOP in the mesenteric adipose tissue. Ossabaw pigs are genetically obese due to the presence of a thrifty phenotype (Lassaletta et al., 2012) that enhances their capacity to deposit substantially more adipose mass compared to their highly selected commercial genotypes. It is presently unknown whether these genetic differences are partly responsible for the differences in the regulation of CHOP protein level during heat stress between the two genotypes.

Heat stress increases IL-6 mRNA level in subcutaneous adipose tissue. IL-6 is highly expressed in adipose tissue where it is positively correlated with obesity in humans (Kwon and Pessin, 2013). Therefore, similar mechanisms might mediate increased mRNA level of IL6 in both obesity and heat stress in adipose tissue. The potential metabolic function of IL6 in heat stress is currently unknown. In obesity, IL-6 interrupts insulin signaling through induction of suppressor of cytokine signaling 3 (SOCS3) in the hepatocyte (Senn et al., 2003). However, IL6 might play a yet undiscovered role in heat stress adaptation because IL-6 knockout mice are intolerant of severe hyperthermia (Phillips et al., 2015). In contrast, TNF-α mRNA level was decreased by

heat stress in subcutaneous fat. This could serve to reduce the level of basal lipolysis in subcutaneous adipose tissue because inhibition of TNF-α has been shown to result in reduction of lipolysis in adipocytes (Souza et al., 1998; Zhang et al., 2002; Souza et al., 2003). In addition, reduction of TNF-α could be a strategy to reduce TNF-α-induced apoptosis in adipocytes (Prins et al., 1997), helping to preserve adipose mass in heat stress. Thus, downregulation of TNF-α may be part of the overall adaptive and survival mechanisms induced during heat stress. The implication of these observed changes on growth performance and composition of gain is still unclear. Although there have been reports that heat stress results in leaner carcasses in pigs (Cruzen et al., 2015; Johnson et al., 2015), our results indicate that heat stress may lead to increased carcass lipid storage, confirming previous reports (Christon, 1988; Kouba et al., 2001; Qu et al., 2016). Although local differences in experimental conditions may explain observed differences lipid deposition during heat stress, the increased mRNA and protein level of PCK1 in adipose tissue in heat stress indicates increased capacity for lipid synthesis. In addition, increased HSP70 in heat stress may result in reduced lipid mobilization. Furthermore, the downregulation of TNFα in adipose tissue during heat stress may limit its lipolytic effect, further helping to preserve adipose tissue. This is similar to the reduction in mRNA level of lipolytic genes in pigs subjected to heat stress (Kellner et al., 2016), resulting in fatter carcasses compared to pair-fed thermoneutral controls. Thus, it is plausible that pigs have developed adaptive and survival mechanisms that help to preserve adipose tissue during heat stress when feed intake is reduced to ensure availability of energy to support critical metabolic processes.

In conclusion, acute and chronic heat stress induce differential adaptive tissue responses. Induction of glyceroneogenic genes such as PCK1 and GK in adipose tissue but not the liver suggests that increased glyceroneogenesis remains a major adaptive response in adipose tissue. This also represents a common adaptive response in the wild unselected Ossabaw pigs and highly lean commercial pigs used in the present study. Overall, these results indicate that increased adipogenic and lipogenic factors such as PCK1, LPL and ER stress marker CHOP may play a major role in the protection and recovery of tissue function in adipose tissue under heat stress in pigs.

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Table 4.1 Feed ingredient composition (as-fed basis).

Ingredient	Percentage
Corn	79.75
Soybean meal	1.95
Dried distiller's grains	15.00
Swine white grease	1.00
L-lysine hydrochloride	0.38
L-threonine[1]	0.09
L-tryptophan	0.04
Limstone	1.14
Monocalcium phosphate	0.14
Vitamin premix[2]	0.10
Sodium chloride	0.25
Selenium 270 premix[3]	0.03
Phytase[4]	0.08
Non-sulfur T.M. premix[5]	0.05
CP, %	12.0
ME, Mcal/kg	3.37

[1] 98% L-threonine. [2]Swine vitamin concentration in premix: vitamin A, 544,680 IU/kg; vitamin D_3, 54,448 IU/kg; vitamin E, 3631 IU/kg; menadione (vitamin K), 182 mg/kg; vitamin B_{12}, 3.2 mg/kg; riboflavin, 726 mg/kg; d-pantothenic acid, 1816 mg/kg; niacin, 2723 mg/kg, biotin, 18.1 mg/kg; folic acid, 136 mg/kg; choline, 45,390 mg/kg; pyridoxine, 409 mg/kg; vitamin E, 1816 IU/kg; chromium, 16.3 mg/kg; carnitine, 4805 mg/kg. [3]Selenium 270 premix: 600 mg/kg of selenium as sodium selenite in a mixture of equal portions of ground limestone and ground corn to fill 0.05% of the diet. [4]600 ppm phytase. [5]Non-sulfur trace mineral premix: iron, 51.05%; zinc, 20.73%; manganese, 2.86%; copper, 1.56%; iodine, 0.046%.

Table 4.2 Primer sequences for real time-PCR.

Gene[1]	Forward	Reverse
18S	5'-ATC CCT GAG AAG TTC CAG CA-3'	5'-CCT CTT GGT GAG GTC GAT GT-3'
aP2	5'-TGG TAC AGG TGC AGA AGT GG -3'	5'-ATT CTG GTA GCC GTG ACA CC -3'
Adiponectin	5'-TTT CTG GGC CCA CTG TGT TT-3'	5'-GGT TTT GCA TTG CAG GCT CA-3'
CD36	5'-ATC GTG CCT ATC CTC TGG-3'	5'-CCA GGC CAA GGA GGT TAA-3'
DGAT2	5'-CAC CTA CTC CTT CGG GGA GA-3'	5'-CTT GGA GTA GGG CAT GAG CC-3'
FAS	5'-AGT TTG TGA TGG AGA ACA CGG CCT -3'	5'-TGT TCA CAC GTG GTG CAA GGG TTA -3'
FATP4	5'-CAT TGT GGC TCA GCA GGT TA-3'	5'-CAG GCT AGG GGT CAA ATC AA-3'
FATP6	5'-TTC TTC GGCTAT GCT GGC AA-3'	5'-TGG ACC ATTAGG TCT CCG GT-3'
GK	5'-CGC TGA GGA AAG TGA AAT CCG -3'	5'-TCG CGT CTT TGG AAT CTA CGA -3'
GLUT4	5'-GAA GGA AGA AGG CAA TGC TG-3'	5'-GAG GAA CCG TCC AAG AAT GA-3'
HSP70	5'-TTC GTG GAC AGA AGC CAC AG -3'	5'-TTG CTA GGA TCT CCA CCC GA -3'
IL-6	5'-TCT GGG TTC AAT CAG GAG ACC TGC-3'	5'-TGC ACG GCC TCG ACA TTT CCC-3'
Leptin	5'-TTG GCC CTA TCT GTC CTA CG-3'	5'-GTG ACC CTC TGT TTG GAG GA-3'
LPL	5'-ATT CAC CAG AGG GTC ACC TG-3'	5'-AGC CCT TTC TCA AAG GCT TC-3'
PCK1	5'-CCC TGC CTT TGA AAA AGC CC -3'	5'-GGA GAT GAT TTC TCG GCG GT -3'
TNF-α	5'-CGT CGC CCA CGT TGT AGC CAA T-3'	5'-GCC CAT CTG TCG GCA CCA CC-3'

[1]aP2=fatty acid binding protein 2; CD36=fatty acid translocase 36; DGAT2=diglyceride acyltransferase 2; FAS=fatty acid synthase; FATP4=fatty acid transport protein 4; FATP6=fatty acid transport protein 6; GK=glycerol kinase; GLUT4=glucose transporter protein type 4; HSP 70=heat shock protein 70; IL-6=interleukin 6; LPL=lipoprotein lipase; PCK1=phosphoenolpyruvate carboxykinase 1; TNF-α=tumor necrosis factors α.

Table 4.3 Effects of heat stress on performance in pigs.

Variable[1]	Control	PF	HS	AHS	SEM	*P*-value[2]	*P*-value[3]
IBW, kg	72.0	72.0	71.9	71.9	0.5	0.991	0.993
FBW, kg	78.8^{a}	74.2^{b}	73.7^{b}	70.5*	0.7	<0.001	0.048
ΔBW, kg	6.8^{a}	2.2^{b}	1.8^{b}	-1.4*	0.6	<0.001	<0.001
IRT, °C	39.74	39.24	39.27	39.66	0.11	0.263	0.452
FRT, °C	39.22	39.05	39.49	39.93	0.08	0.543	0.213
ΔRT, °C	-0.52^{b}	-0.19^{ab}	0.22^{a}	0.27	0.13	0.043	0.731

[1]IBW=initial body weight; FBW=final body weight; ΔBW=body weight change; IRT=initial rectal temperature; FRT=final rectal temperature; ΔRT=rectal temperature change; Different subscripts represent differences between Control, pair-fed, and heat stress at $P< 0.05$. Asterisk represent differences between heat stress and acute heat stress at $P< 0.05$. [2]7 day treatments (CON, PF and HS). [3]High temperature treatments (HS and AHS). (n=10 pigs/treatment).

Table 4.4 Effects of heat stress on subcutaneous fat mRNA level in pigs.

Gene[1]	Control	PF	HS	AHS	SEM	*P*-value[2]	*P*-value[3]
HSP70	1.04[b]	0.67[b]	1.79[a]	1.71	0.14	0.003	0.836
PCK1	0.64[b]	0.56[b]	3.3[a]	1.22[*]	0.24	<0.001	<0.001
GK	1.29	0.97	1.3	0.77[*]	0.07	0.258	0.003
FAS	1.01	1.18	1.22	1.15	0.08	0.454	0.694
LPL	1.22	1.05	1.25	0.88	0.08	0.672	0.233
Adiponectin	2.06	1.07	1.11	1.13	0.06	0.889	0.972
Leptin	1.60	0.81	1.49	1.07	0.12	0.055	0.313
aP2	1.10[ab]	0.83[b]	1.39[a]	1.08	0.06	0.035	0.362
FATP4	1.17	0.94	2.12	2.01	0.20	0.282	0.742
FATP6	1.24	0.92	1.29	1.04	0.08	0.414	0.578
GLUT4	1.54[a]	1.09[ab]	1.34[a]	0.70[*]	0.10	0.046	0.011
DGAT2	1.42	1.09	1.49	0.88	0.12	0.692	0.077
IL-6	0.76[b]	0.88[b]	2.23[a]	1.30	0.15	<0.001	0.051
TNF-α	1.96[a]	0.76[b]	1.24[b]	1.30	0.13	<0.001	0.848

[1] HSP 70=heat shock protein 70; PCK1=phosphoenolpyruvate carboxykinase 1; GK=glycerol kinase; FAS=fatty acid synthase; LPL=lipoprotein lipase; aP2=fatty acid binding protein 2; FATP4=fatty acid transport protein 4; FATP6=fatty acid transport protein 6; GLUT4=glucose transporter protein type 4; DGAT2=diglyceride acyltransferase 2 IL-6=interleukin 6; TNF-α=tumor necrosis factors α. Different subscripts represent differences between Control, pair-fed, and heat stress at $P< 0.05$. Asterisk represent differences between heat stress and acute heat stress at $P< 0.05$. [2]7 day treatments (CON, PF and HS). [3]High temperature treatments (HS and AHS). (n=10 pigs/treatment).

Table 4.5 Effects of heat stress on mesenteric fat gene expression in pigs.

Gene[1]	Control	PF	HS	AHS	SEM	*P*-value[2]	*P*-value[3]
HSP70	0.84	1.66	1.07	1.74	0.17	0.361	0.212
PCK1	0.63[b]	1.00[b]	3.38[a]	1.27[*]	0.27	<0.001	0.039
GK	1.32	1.55	1.62	0.87	0.16	0.869	0.295
FAS	0.96	1.08	1.11	1.66	0.11	0.305	0.278
LPL	1.64	1.28	1.28	0.64[*]	0.12	0.289	0.036
CD36	1.32	1.55	1.62	0.87	0.16	0.785	0.186
Adiponectin	1.40	1.23	1.04	1.28	0.12	0.356	0.742
Leptin	2.05[a]	0.82[b]	1.58[ab]	1.01	0.15	0.012	0.083
aP2	1.33	1.02	1.24	1.17	0.10	0.727	0.603
FATP4	1.03	0.99	1.30	1.20	0.11	0.301	0.858
FATP6	1.03	1.19	1.82	1.00	0.14	0.091	0.319
GLUT4	1.32	1.56	1.55	1.39	0.10	0.083	0.759
DGAT2	1.72	1.24	1.52	0.76	0.12	0.395	0.256
IL-6	1.02	1.53	1.01	1.56	0.13	0.251	0.631
TNF-α	1.47	1.16	0.87	1.22	0.11	0.162	0.096

[1] HSP 70=heat shock protein 70; PCK1=phosphoenolpyruvate carboxykinase 1; GK=glycerol kinase; FAS=fatty acid synthase; LPL=lipoprotein lipase; CD36=fatty acid translocase 36; aP2=fatty acid binding protein 2; FATP4=fatty acid transport protein 4; FATP6=fatty acid transport protein 6; GLUT4=glucose transporter protein type 4; DGAT2=diglyceride acyltransferase 2 IL-6=interleukin 6; TNF-α=tumor necrosis factors α. Different subscripts represent differences between Control, pair-fed, and heat stress at $P< 0.05$. Asterisk represent differences between heat stress and acute heat stress at $P< 0.05$. [2]7 day treatments (CON, PF and HS). [3]High temperature treatments (HS and AHS). (n=10 pigs/treatment).

Table 4.6 Effects of heat stress on expression of genes in the STD1 muscle of pigs.

Gene[1]	Control	PF	HS	AHS	SEM	*P*-value[2]	*P*-value[3]
HSP70	0.37[b]	0.56[b]	3.37[a]	1.18	0.50	<0.001	0.082
PCK1	2.10	1.76	2.25	0.75	0.28	0.584	0.129
GK	1.31	1.01	0.87	1.11	0.07	0.263	0.462
FAS	0.97	1.13	0.94	1.24	0.06	0.667	0.072
LPL	0.87[b]	1.51[b]	4.38[a]	0.66[*]	0.388	<0.001	<0.001

[1]HSP 70=heat shock protein 70; PCK1=phosphoenolpyruvate carboxykinase 1; GK=glycerol kinase; FAS=fatty acid synthase; LPL=lipoprotein lipase STD=semitendinosus muscle; Different subscripts represent differences between Control, pair-fed, and heat stress at $P< 0.05$. Asterisk represent differences between heat stress and acute heat stress at $P< 0.05$. [2]7 day treatments (CON, PF and HS). [3]High temperature treatments (HS and AHS). (n=10 pigs/treatment).

Table 4.7 Effects of heat stress on mRNA level of genes in the LD muscle of pigs.

Gene[1]	Control	PF	HS	AHS	SEM	*P*-value[2]	*P*-value[3]
HSP70	1.10[b]	1.09[b]	3.78[a]	0.92[*]	0.49	0.048	0.003
PCK1	1.25	1.73	1.96	1.06	0.26	0.852	0.383
GK	1.46	1.10	1.01	1.13	0.11	0.092	0.856
FAS	0.97	1.15	1.26	0.73[*]	0.09	0.267	0.034
LPL	1.13	1.28	0.92	0.79	0.11	0.735	0.586

[1]HSP 70=heat shock protein 70; PCK1=phosphoenolpyruvate carboxykinase 1; GK=glycerol kinase; FAS=fatty acid synthase; LPL=lipoprotein lipase; LD=longissimus dorsi muscle. Different subscripts represent differences between Control, pair-fed, and heat stress at $P< 0.05$. Asterisk represent differences between heat stress and acute heat stress at $P< 0.05$. [2] 7 day treatments (CON, PF and HS). [3]High temperature treatments (HS and AHS). (n=10 pigs/treatment).

Table 4.8 Effects of heat stress on mRNA level in the liver of pigs.

Variable[1]	Control	PF	HS	AHS	SEM	*P*-value[2]	*P*-value[3]
HSP70	1.19	0.96	0.90	1.66	0.25	0.757	0.286
PCK1	0.77[b]	2.52[a]	1.64[b]	1.97	0.30	0.047	0.374
GK	1.29	0.90	1.34	1.47	0.12	0.863	0.945
FAS	1.51	1.04	1.11	1.20	0.12	0.283	0.734
LPL	1.52	1.33	1.53	0.99	0.19	0.825	0.093
FATP4	1.50	0.83	1.00	1.68	0.29	0.163	0.265
CD36	1.19	1.42	0.83	1.68	0.16	0.682	0.135
IL-6	1.33	1.05	1.07	0.86	0.21	0.670	0.312
TNF-α	1.49	0.89	1.39	0.99	0.13	0.573	0.263

[1] HSP 70=heat shock protein 70; PCK1: phosphoenolpyruvate carboxykinase 1; GK: glycerol kinase; FAS: fatty acid synthase; LPL=lipoprotein lipase; FATP4=fatty acid transport protein 4; CD36=fatty acid translocase 36; IL-6=interleukin; TNF-α=tumor necrosis factors α. Different subscripts represent differences between Control, pair-fed, and heat stress at $P< 0.05$. Asterisk represent differences between heat stress and acute heat stress at $P< 0.05$. [2]7 day treatments (CON, PF and HS). [3]High temperature treatments (HS and AHS). (n=10 pigs/treatment).

Figure 4.1 Heat stress (HS) effects on performance pigs exposed to either acute or chronic heat stress.

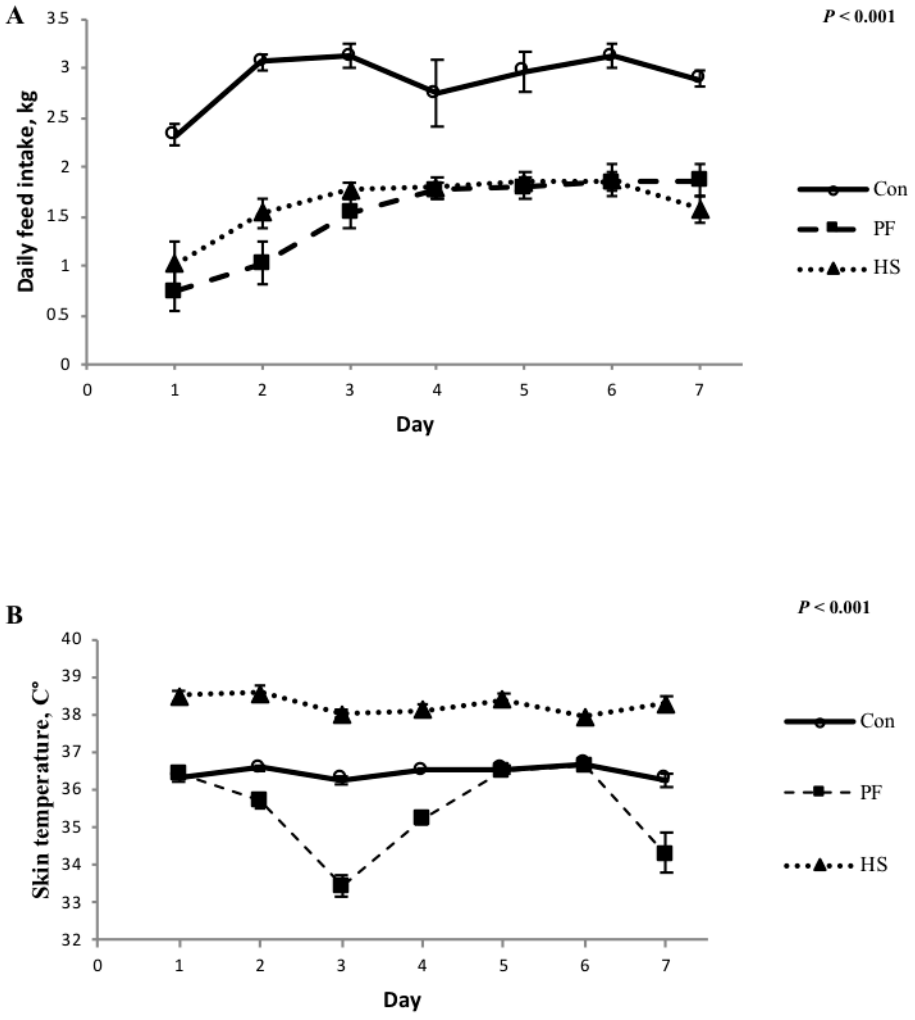
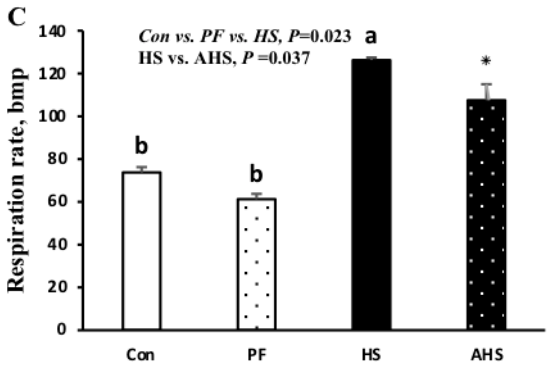


Figure 4.1 continued



Average daily feed intake (A), skin temperature (B), respiration rate (C) in control (Con, 20 °C with ad libitum feed intake for 7 d), pair-fed (PF, 20°C with pair feeding to heat stress treatment for 7d), and heat stress (35°C with ad libitum FI for 7d) environments, and acute heat stress (35°C with ad libitum FI for 24hr) environments. P values in A and B represent the significance of effect of the 7-d treatments. Bars represent means ± SEM. Asterisks a and b represent differences between heat stress and acute heat stress at $P< 0.05$. (n=10 pigs/treatment).

Figure 4.2 Heat stress (HS) effects on plasma metabolites.

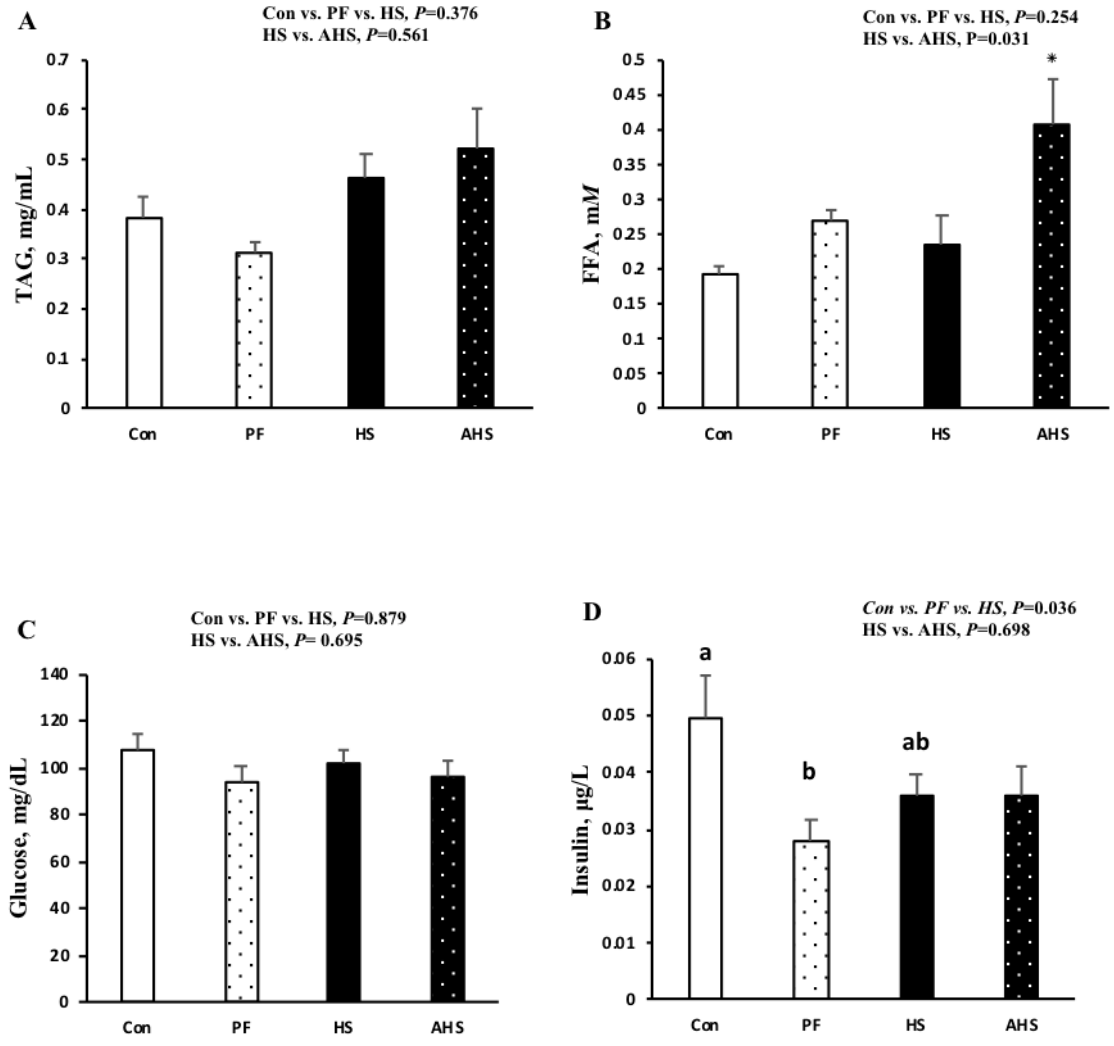
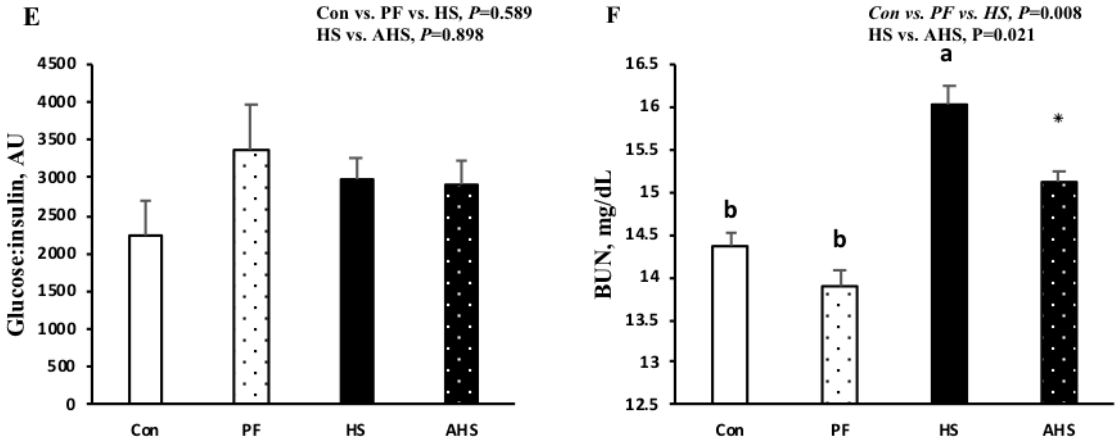


Figure 4.2 continued



Control (Con, 20 °C with ad libitum feed intake for 7 d), pair-fed (PF, 20°C with pair feeding to heat stress treatment for 7d), and heat stress (35°C with ad libitum FI for 7d) environments, and acute heat stress (35°C with ad libitum FI for 24hr) environments. Triacylglycerol (TAG) (A), free fatty acid (FFA) (B), glucose (C), insulin (D), insulin to glucose ration (E), blood urea nitrogen (BUN) (F). Bars represent means ± SEM. $^{a\text{-}b}P<0.05$. Asterisk represent differences between heat stress and acute heat stress at $P< 0.05$.

Figure 4.3 Heat stress (HS) effects on protein levels in subcutaneous fat.

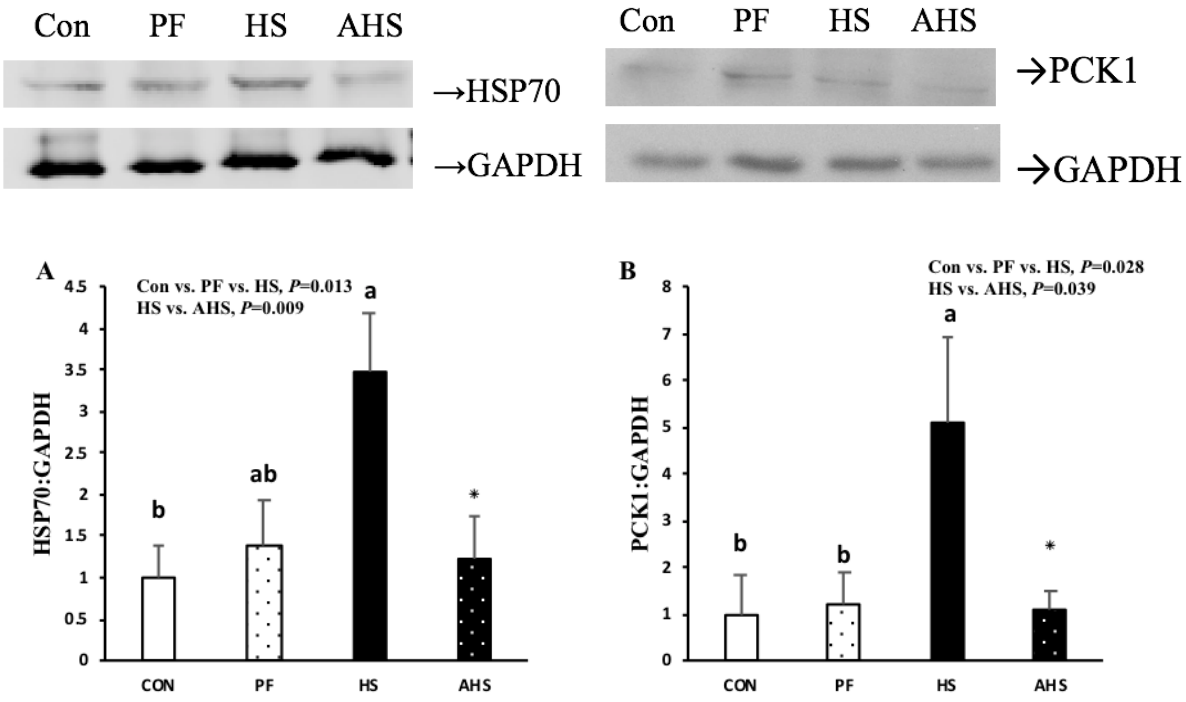
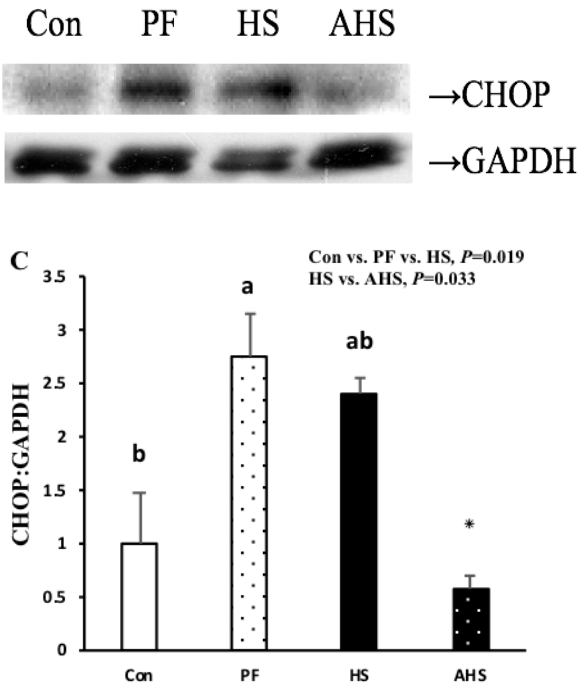


Figure 4.3 contiuned



Control (Con, 20 °C with ad libitum feed intake for 7 d), pair-fed (PF, 20°C with pair feeding to heat stress treatment for 7d), and heat stress (35°C with ad libitum FI for 7d) environments, acute heat stress (35°C with ad libitum FI for 24hr) environments. (A) Heat shock protein 70 (HSP70). (B) Phosphoenolpyruvate carboxykinase (PCK1). (C) CCAAT-enhancer-binding protein homologous protein (CHOP). Bars represent means ± SEM. $^{a\text{-}b}P<0.05$. Asterisk represent differences between heat stress and acute heat stress at $P< 0.05$.

Figure 4.4 Heat stress (HS) effects on protein levels in mesenteric fat.

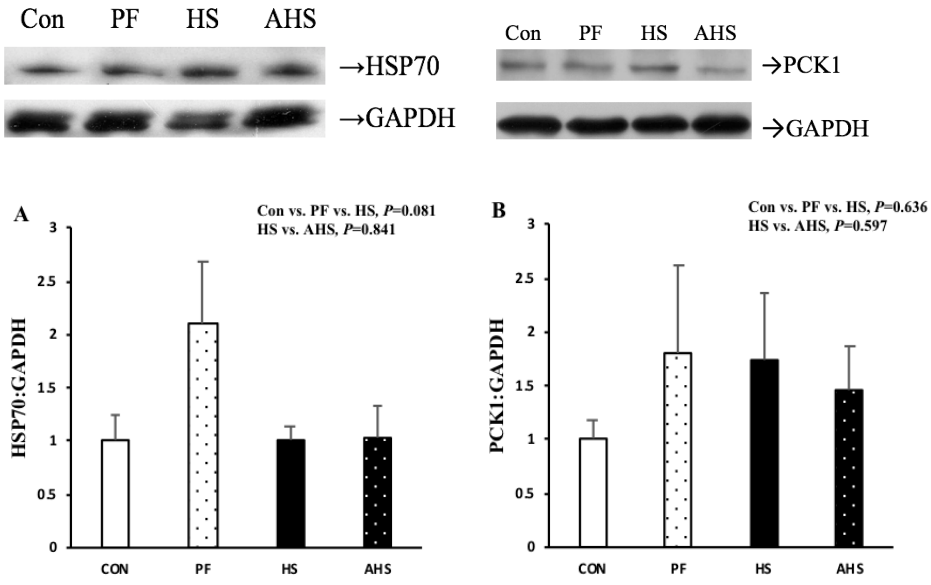
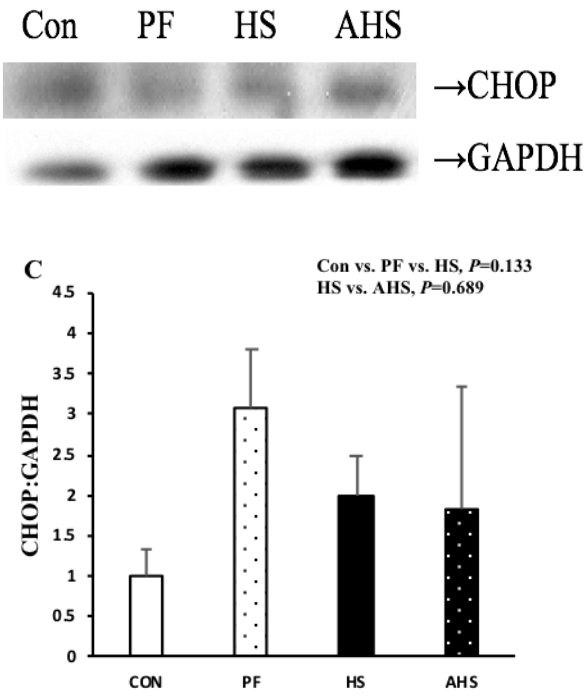


Figure 4.4 Contiuned



Control (Con, 20 °C with ad libitum feed intake for 7 d), pair-fed (PF, 20°C with pair feeding to heat stress treatment for 7d), and heat stress (35°C with ad libitum FI for 7d) environments, acute heat stress (35°C with ad libitum FI for 24hr) environments. (A) Heat shock protein 70 (HSP70). (B) Phosphoenolpyruvate carboxykinase (PCK1). (C) CCAAT-enhancer-binding protein homologous protein (CHOP). Bars represent means ± SEM. $^{a\text{-}b}P<0.05$. Asterisk represent differences between heat stress and acute heat stress at $P< 0.05$.

Figure 4.5 Heat stress (HS) effects on protein levels in liver.

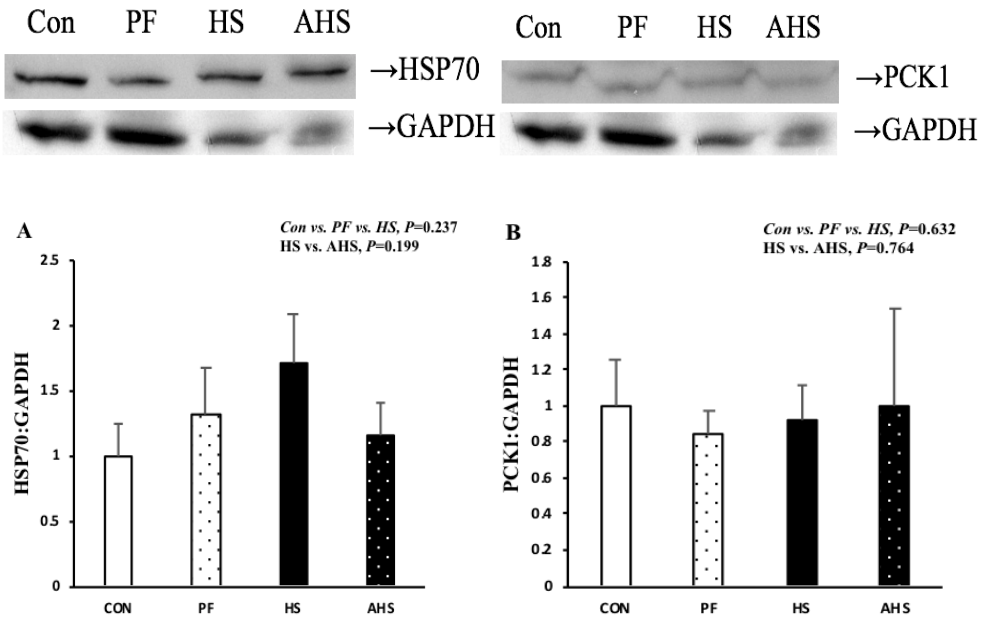
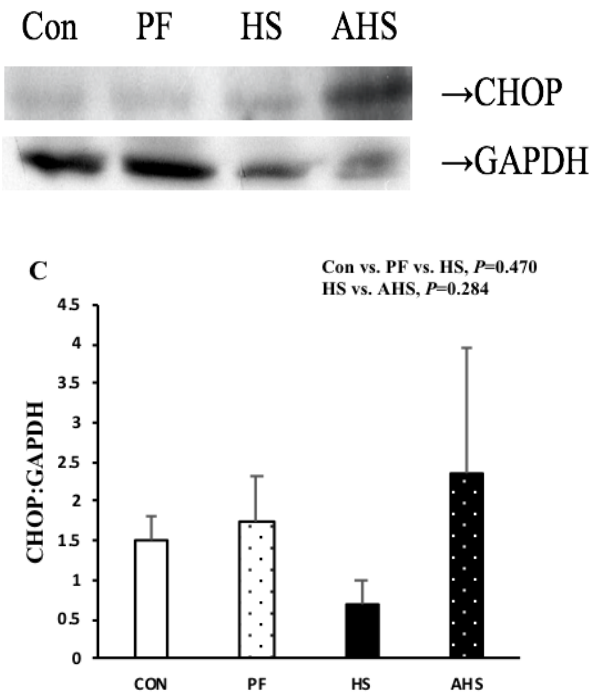


Figure 4.5 Continued



Control (Con, 20 °C with ad libitum feed intake for 7 d), pair-fed (PF, 20°C with pair feeding to heat stress treatment for 7d), and heat stress (35°C with ad libitum FI for 7d) environments, acute heat stress (35°C with ad libitum FI for 24hr) environments. (A) Heat shock protein 70 (HSP70). (B) Phosphoenolpyruvate carboxykinase (PCK1). (C) CCAAT-enhancer-binding protein homologous protein (CHOP). Bars represent means ± SEM. $^{a\text{-}b}P<0.05$. Asterisk represent differences between heat stress and acute heat stress at $P< 0.05$.

Figure 4.6 Heat stress (HS) effects on protein levels in longissimus dorsi muscle.

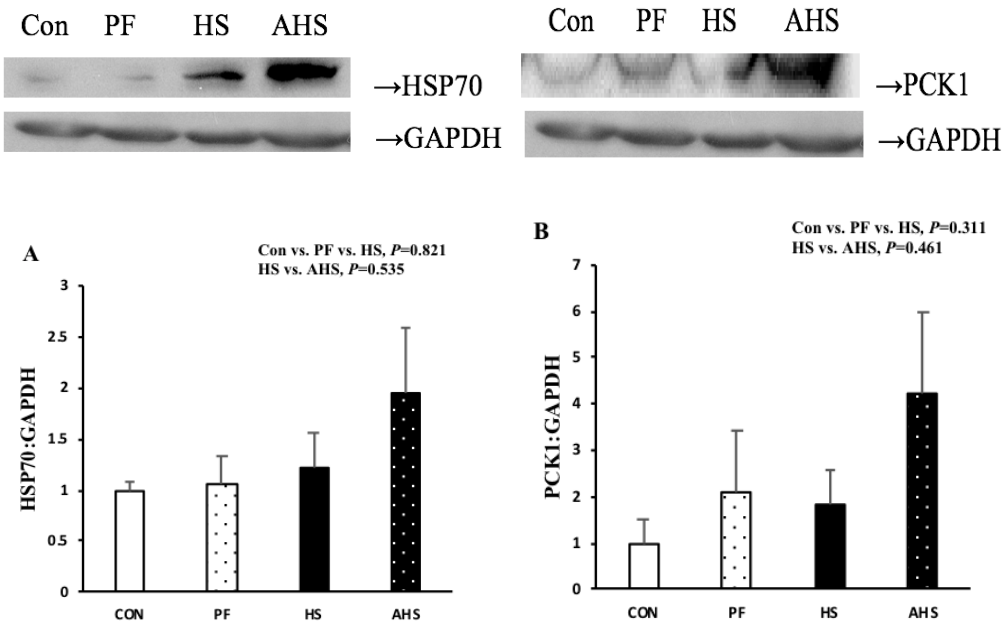
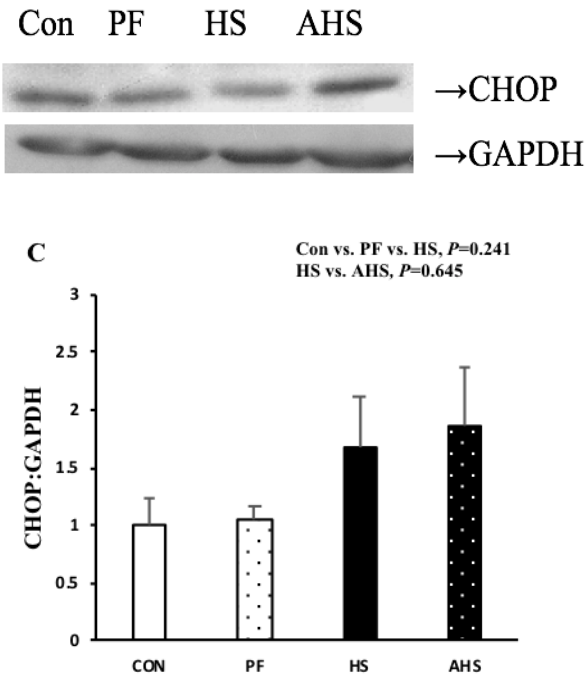


Figure 4.6 Continued



Control (Con, 20 °C with ad libitum feed intake for 7 d), pair-fed (PF, 20°C with pair feeding to heat stress treatment for 7d), and heat stress (35°C with ad libitum FI for 7d) environments, acute heat stress (35°C with ad libitum FI for 24hr) environments. (A) Heat shock protein 70 (HSP70). (B) Phosphoenolpyruvate carboxykinase (PCK1). (C) CCAAT-enhancer-binding protein homologous protein (CHOP). Bars represent means ± SEM. $^{a\text{-}b}P<0.05$. Asterisk represent differences between heat stress and acute heat stress at $P< 0.05$.

CHAPTER 5. CYTOSOLIC PHOSPHOENOLPYRUVATE CARBOXYKINASE IS A RESPONSE GENE INVOLVED IN PORCINE ADIPOCYTE ADAPTATION TO HEAT STRESS

5.1 Abstract

Prolonged exposure to high temperature leads to increased lipid storage and gene and protein level of cytosolic phosphoenolpyruvate carboxykinase (PCK1) in pig adipocytes. However, the importance of PCK1 activation and lipid storage in the adaptive response to heat stress is unknown. Therefore, in vitro experiments were conducted to investigate the effect of PCK1 inhibition with 3-mercaptopicolinic acid (3MPA) on lipid storage and adipocyte response during HS. In vitro culture of adipocytes under heat stress (41.0 °C) increased ($P < 0.05$) triacylglycerol (TAG) accumulation compared with control (37.0 °C). Heat stress increased ($P < 0.05$) reactive oxygen species (ROS) level and 3MPA further upregulated ($P < 0.05$) its level. Heat shock protein 70 (HSP70) gene expression was induced ($P < 0.05$) by HS compared to control, and PCK1 inhibition with 3MPA attenuated ($P < 0.05$) its induction by HS. The ER stress markers, C/EBP homologous protein (CHOP) was also upregulated by HS and 3MPA further upregulated ($P < 0.05$) CHOP mRNA level. These results suggest that with inhibition of PCK1 during HS, in vitro cultured adipocytes were less able to induce adaptive responses such as upregulation of HSP70 and triglycerides, and this exacerbated ER stress during HS. Thus, PCK1 may function to alleviate ER stress that occurs during HS.

Keywords: adipocyte, ER stress, heat stress, PCK1 or PEPCK

5.2 Introduction

High temperature induces systemic and cellular response that lead to metabolic change aimed at heat stress adaptation. However, the mechanisms of systemic, organ and cellular level adaptive response to heat stress are still not well understood. In our previous study in vitro (Qu et al., 2015), we showed that, despite hypercatabolic effects of heat stress, pig adipocytes have upregulated lipogenesis in a cell autonomous manner, with the resulting increase in cellular

triglyceride concentration. Studies have shown that heat stress resulted in increased carcass fat in pig (Bridges, 1998; Collin et al., 2001), through induction of LPL (Kouba et al., 2001) and perhaps increased insulin signaling (Pearce et al., 2013). We also reported that heat stress led to an upregulation of glyceroneogenesis markers, cytosolic phosphoenolpyruvate carboxykinase (PCK1/PEPCK-C) (Qu et al., 2016) and to a limited extent, glycerol kinase (GK). However, cytosolic PCK1, is the key glyceroneogenesis enzyme in the adipocyte and catalyzes the conversion of oxaloacetate to phosphoenolpyruvate (PEP) (Chang et al., 1966). At present, it is unclear whether PCK1 plays any role in the adaptive response to heat stress, although glycerol, a metabolic product of PCK1, is a known chemical chaperon and may play an important role during heat stress response. The use of 3-mercaptopicolinic acid (3MPA) as a gluconeogenesis inhibitor, through inhibition of PCK1, has been reported previously (Jomain-Baum et al., 1976). This inhibitor acts as a non-competitive inhibitor of PCK1 with respect to oxaloacetate and Mn^{2+}.

Therefore, the objective of this study was to determine the response of adipocytes to HS when the enzyme activity of PCK1 was inhibited with 3MPA.

5.3 Materials and Methods

Primary cell isolation and cell culture

The Purdue Animal Care and Use Committee approved all animal care and use protocols described in these experiments. Isolation and culture of primary porcine preadipocytes were based on the protocol described previously (Qu et al., 2015). Briefly, preadipocytes/stromovascular cells (SVC) were isolated from the inner layer of subcutaneous adipose tissue from neonatal male piglets (less than 7 d old). Excised adipose tissue was immediately kept in buffered saline (0.15 M NaCl, 10 mM HEPES, pH 7.4) at 37 °C. The tissue was minced with a pair of scissors and then incubated with a digestion cocktail (10 mM NaHCO3, 10 mM HEPES, 5 mM D-glucose, 120 mM NaCl, 4.6 mM KCl, 1.25 mM CaCl2, 1.20 mM MgSO4, 1.20 mM KH2PO4, 3% BSA) containing collagenase (Cat. # CLS1, collagenase type I, 1mg/mL, Worthington Biochemical Corp., Lakewood, NJ) in a shaking water bath for 45 min at 120 oscillation/min at 37 °C. When thoroughly digested, sample was centrifuged at 2,000 × g at 22°C for 10 min to separate the floating adipocytes from the precipitated SVC pellet. The

SVC was washed twice in the digestion cocktail. Afterwards, the pellet was suspended in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) medium (Cat. # D8900, Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum (Cat. # 35010CV, Mediatech, Manassas, VA), 1% antibiotic-antimycotic (Cat. # A5955, Sigma Aldrich, St. Louis, MO), plated in sterile 24-well cell culture plates and incubated in a humidified incubator with 5% CO_2 and 95% air. Adipocytes from three pigs were pooled. Experiments were conducted with several replicates of these pooled cells. Upon reaching confluence (approx. after 4-5 d), cells were differentiated in a differentiation medium (DMEM/F12 with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic, 1μM insulin, 1μM dexamethasone (1mM), 1μM rosiglitazone, 1 μM biotin, 1μM triiodothyronine (T3), 1 μM pantothenic acid). Media was replaced every 3d and rosiglitazone was removed from the differentiation media after d 3 of differentiation. Cells were treated with 0.05 mM of the PCK1 inhibitor, 3MPA, (Cat. # 320386-54-7, Santa Cruz Biotechnology, Santa Cruz, CA) dissolved in DMSO or DMSO control on d7 of differentiation and set at either 37°C (normal) or high (41.5°C) temperature conditions. The treatment medium was changed after 2 d. The experiment was terminated on d9 of differentiation. Each experiment was repeated 6 different times (replicates).

mRNA level *analysis*

Total RNA was extracted from cells using the QIAzol lysis reagent (Cat. # 79306, Qiagen, Valencia, CA). Extracted RNA was then dissolved in nuclease-free water (Cat. # AM9906, Ambion, Austin, TX). The concentration of extracted RNA was measured on a Nanodrop 1000 instrument (Thermo Scientific, Waltham, MA). Integrity of RNA and genomic DNA contamination were checked by electrophoresis on 0.8% agarose with ethidium bromide staining. One μg RNA was reverse transcribed with moloney murine leukemia virus (MMLV) reverse transcriptase (Cat. # M1701, Promega, Madison, WI). PCR assay was done on a Bio-Rad MyiQ thermocycler (Bio-Rad, Temecula, CA). The PCR reaction mixtures contained 0.5 μg of cDNA, 0.075 nmol of forward and reverse primers respectively, and RT2 SYBR Green qPCR master mix (Cat. # 330513, Qiagen, Valencia, CA) in a total reaction volume of 20 μL. The reaction mix was incubated for 5 min at 95°C for the initial denaturation step, and then 40 cycles of the following steps: 10 s at 95°C, 20 s at 55°C, and 20 s at 72°C. RT-PCR primer sequences are

presented in Table 1. Abundance of mRNA for each gene was calculated after its cycle threshold (Ct) was normalized to the Ct for 18S using the ΔΔCt method. Samples were analyzed in duplicate.

Immunoblotting analysis

Cells were rinsed free of medium twice with cold 1 × phosphate buffered saline (PBS) and then suspended in 1 × radio-immunoprecipitation assay (RIPA) buffer (10% Nonidet P-40, 0.5 M Tris-HCl, 2.5% deoxycholic acid, 1.5 M HCl, and 10 mM ethylene diamine tetra acetic acid, EDTA) supplemented with commercial protease and phosphatase inhibitor cocktails (Cat. # P8340 and Cat. # P2850, Sigma-Aldrich, St. Louis, MO). Protein concentrations were measured with the bicinchoninic acid assay reagent (Cat. # BCA1, Sigma-Aldrich, St. Louis, MO). Proteins (40 μg per sample) were resolved on a 10% SDS polyacrylamide gel and then transferred to nitrocellulose membranes (Cat. #1620112, Bio-Rad, Temecula, CA). Thereafter, membranes were blotted with primary antibodies: anti-β-actin (Cat. # 4970, Cell Signaling Technology, Danvers, MA), HSP70 (Cat. # Cayman Chemicals, Ann Arbor, MI), anti-HSP90 (Cat. # 4877, Cell Signaling Technology), anti-CCAT/enhancer-binding homologous protein (CHOP) (Cat. # 2895, Cell Signaling Technology, Danvers, MA), phospho-AMPK (AMP activated protein kinase) (Cat. # 2535, Cell Signaling Technology), AMPK (Cat. # 2532, Cell Signaling Technology), phospho- eukaryotic initiation factor 2 (EIF2α) (Cat. # 3398, Cell Signaling Technology), EIF2α (Cat. # 9722, Cell Signaling Technology). Secondary antibodies used were horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit IgG (Cat. # 7074, Cell Signaling Technology). Membranes were developed with Immobilon HRP substrate (Cat. # WBKLS0500, Millipore, Billerica, MA) and exposed to autoradiographic film (Cat. # sc-201697 Santa Cruz Biotechnology, Dallas, TX). Band intensities were quantified with a Kodak 1 D 3.6 imaging software (Kodak, Rochester, NY). Signal intensity of a specific protein was normalized to that of β-actin for each sample. Sample were analyzed once.

Triglyceride (TAG) and free glycerol determination

Adipocytes were washed three times with warm PBS. Total intracellular lipids, including triacylglycerol (TAG), was extracted with isopropanol for 30 min at room temperature with

gentle agitation. Thereafter, TAG content was measured using a TAG determination kit (Cat. # TR0100, Sigma-Aldrich) according to the manufacturer's instructions. Lipolysis was determined by measuring media glycerol concentration in unstimulated cells or cells stimulated with 10μM norepinephrine (Cat. # A7257, Sigma-Aldrich) for two hours. Triglyceride and free glycerol content were normalized to cellular protein content.

Measurement of cellular ATP

Concentration of ATP was determined in total cellular lysate recovered with 1 × RIPA buffer. Levels of ATP were measured with an ATP determination kit (Cat. # A22066, Invitrogen, Carlsbad, California). Intracellular ATP amount was normalized to total cellular protein.

Measurement of cellular reactive oxygen species (ROS)

Cells were washed with PBS and then loaded with ROS sensitive dye, CM-H2DCFDA (Cat. # C6827, Thermo Fisher Scientific, St. Louis, MO). Thereafter, cellular ROS level was determined according to the manufacturer's protocol.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay

Adipocyte viability was determined with the MTT assay. After treatment of adipocytes in 96-well plates, medium was replaced with fresh medium containing MTT (Cat. # TOX1, Sigma-Aldrich, St. Louis, MO) and viability was determined based on the manufacturer's protocol.

Statistical analyses

Data were analyzed as a two-way factorial analysis of ANOVA with temperature (37 or 41.5 °C) and PCK1 inhibition treatment (DMSO control or 3MPA) as the main factors. These main factors were considered fixed effects, whereas replicate was considered a random effect. Tukey's multiple comparison analysis was used to separate the means when interaction effect was significant according to the procedures of SAS (SAS Inst. Inc., Cary, NC). Differences were considered significant with $P < 0.05$, and P-values between 0.05 and 0.10 were considered as showing a strong tendency of significance. Bars in the figures represent means ± SEM.

5.4 Results

Effect of heat stress and PCK1 inhibition on lipogenesis

To determine the effect of PCK1 inhibition on lipid storage during in vitro HS, cellular TAG concentration was measured in cells treated with 3MPA. High temperature induced TAG accumulation in the adipocytes compared with control, and 3MPA treatment numerically reduced (by approx. 20%) the TAG concentration under heat stress ($P < 0.05$) (Figure 5.1A). However, there was no further reduction of TAG level by 3MPA under control temperature. To determine the effect of heat on the viability of adipocyte in vitro, we performed the MTT assay. Although heat had no effect on viability compared to control, inhibition of PCK1 enzymatic activity with 3MPA treatment resulted in significantly lower ($P < 0.05$) (by approx. 18 %) cell viability than DMSO-treated cells in both in 37 and 41.5 ℃ temperature conditions (Figure 5.1B).

Effect of heat stress and PCK1 inhibition on lipogenesis markers

To determine effects of high temperature and PCK1 inhibition on lipogenesis markers in adipocyte differentiation in vitro, we measured mRNA level of key lipogenic enzymes. mRNA level of PCK1 was significantly elevated ($P < 0.05$) by heat stress treatment compared to control (Figure 5.2A). Moreover, 3MPA significantly suppressed ($P < 0.05$) PCK1 mRNA level in control and HS cells. mRNA level of lipoprotein lipase (LPL) was induced ($P < 0.05$) by HS (Figure 5.2B), but 3MPA treatment only tended ($P < 0.08$) to downregulate its mRNA level in both control and high temperature conditions. There was a tendency ($P < 0.09$) for mRNA level of fatty acid synthase (FAS) to be induced under high temperature conditions (Figure 5.2C). Inhibition of PCK1 with 3MPA downregulated ($P < 0.05$) FAS mRNA level in both control and HS condition. mRNA level of SREBP-1c was induced by HS ($P < 0.05$), but 3MPA did not affect its mRNA level (Figure 5.2D). mRNA level of DGAT2 was upregulated by HS treatment ($P < 0.05$), but there was no effect of 3MPA (Figure 5.2E).

Effect of heat stress and PCK1 inhibition on lipolysis

To determine whether high temperature and PCK1 affect lipolysis, we measured free glycerol concentrations in the cell culture media in unstimulated (basal lipolysis) or norepinephrine-stimulated cells. There was no difference in basal lipolysis in cells in control or HS temperature condition (Figure 5.3A). However, 3MPA significantly suppressed ($P < 0.05$) basal lipolysis in cells under HS. In norepinephrine-stimulated cells (Figure 5.3B), there was a tendency ($P < 0.07$) for reduced free media glycerol concentration in HS compared to control. Treatment with 3MPA significantly ($P < 0.05$) suppressed lipolysis in both control and cells under HS (Figure 5.3B). To determine whether HS regulates mRNA level of an important lipolytic gene, we measured the mRNA level of adipose triglyceride lipase (ATGL) by PCR. mRNA level of ATGL was significantly induced ($P < 0.05$) by HS compared with control temperature (Figure 5.3C), and 3MPA downregulated ($P < 0.05$) its mRNA level in cells under both control and HS conditions. To further determine whether HS and PCK1 inhibition affected the mRNA level is involved in glycerol transport, we measured the mRNA level of aquaporin7 (AQP7), a protein that is involved in glycerol trafficking that is abundantly expressed in adipocytes (Jin et al., 2014). Although HS induced ($P < 0.05$) AQP7 mRNA level than in control cells, 3MPA treatment had no effect on its mRNA level (Figure 5.3D).

Effect of heat stress and PCK1 inhibition on metabolic markers and cellular ATP level

mRNA level of beta-oxidation markers, acyl-CoA thioesterase 1 (ACOT1) was not affected by HS or 3MPA (Figures 5.4A). There was a significant ($P < 0.05$) temperature × treatment interaction effect on the mRNA level of carnitine palmitoyl transferase 1 (CPT1) such that control cell treated with 3MPA had the highest mRNA level, whereas its mRNA level was lowest in HS cells treated with this inhibitor. To investigate whether heat stress had effects on glucose metabolism, we measured mRNA level of pyruvate dehydrogenase kinase 4 (PDK4) and lactate dehydrogenase (LDH). Both PDK4 and LDH were induced ($P < 0.05$) by HS and 3MPA had no effects on their mRNA level (Figures 5.4C and D). Heat stress significantly increased ($P < 0.05$) ATP concentration (Figures 5.4E) and 3MPA treatment upregulated ($P < 0.05$) ATP concentration in cells under both control and HS temperature conditions. We also measured the cellular levels of phosphorylated and total AMPK as markers of cellular energy status. Heat

stress treatment tended ($P < 0.1$) to increase phospho-AMPK/AMPK. However, there was a significant ($P < 0.05$) interaction effect on phospho-AMPK/AMPK ratio such that control cells treated with 3MPA treatment had the same level of phospho-AMPK/AMPK ratio as the two HS treatments, whereas control cells without the inhibitor had the lowest ratio (Figures 5.4F).

Effect of heat stress and PCK1 inhibition on ER stress markers

To determine whether heat stress regulated ER stress in in vitro differentiated adipocytes, we measured mRNA and protein level of ER and heat stress markers. Heat stress tended ($P < 0.08$) to increase ROS concentration as indicated by the fluorescence of the ROS sensitive CM-H2DCFDA dye (Figure 5.5A) and 3MPA further upregulated ($P < 0.05$) its level under both control and HS conditions. Additionally, HS increased ($P < 0.05$) heat shock protein 70 (HSP70) mRNA level compared to control (Figure 5.5B). Treatment with 3MPA downregulated ($P < 0.05$) HSP70 protein level only in HS cells (Figure 5.5B). Inhibition of PCK1 with 3MPA upregulated HSP70 protein in cells in control temperature ($P < 0.05$) but decreased it under HS ($P < 0.05$) (Figure 5.5C). Heat stress also tended ($P < 0.09$) to induce HSP90 mRNA level but had no significant effects on HSP90 protein level (Figure 5.5D and E) and 3MPA increased ($P < 0.05$) its mRNA abundance in both control and HS cells (Figure 5.5D). Heat stress under induced ($P < 0.05$) the mRNA level of other stress markers, ATF6 and SOD1, ATF4 and p-eIF2α compared to control (Figures 5.6A and B), and 3MPA further increased ($P < 0.05$) their mRNA level in both control and HS cells. mRNA level of ATF4 was induced ($P < 0.05$) by HS, but not affected by 3MPA (Figure 5.6C). The mRNA level of ER stress markers, C/EBP homologous protein (CHOP) and XBP1 (Figures 5.7A and C) were induced ($P < 0.05$) in HS and further upregulated ($P < 0.05$) by 3MPA in both control and HS conditions. Protein abundance of CHOP (Figure 5.7B) was not affected by either heat stress or 3MPA. mRNA level of GRP78 was induced only by HS (Figure 5.7 D), but there was no effect of 3MPA.

5.5 Discussion

Adipocytes exposed to HS in vitro respond by increasing cellular triglyceride storage (Qu et al., 2015). We have demonstrated previously (Qu et al., 2015) and in this experiment that this response is accompanied by increased mRNA level of lipogenic genes such as LPL, PCK1, PPARγ, and SREBP1c, FAS and DGAT2. Previous in vivo studies have shown that heat stress resulted in increased carcass fat in pigs (Bridges, 1998; Collin et al., 2001), through induction of LPL (Kouba et al., 2001) and insulin signaling pathway (Pearce et al., 2013). Our previous experiment also confirmed induction of numerous lipogenic genes such as PCK1 and LPL in the subcutaneous fat depot of pigs exposed to heat stress for 7 days (Qu et al., 2016). Thus, although the precise mechanisms are still not completely known, heat adaptation involves increased lipogenesis in pigs, and this response is always accompanied by increased PCK, suggesting that this gene might play a critical role in the increased lipid storage in adipocytes during heat stress. We have further demonstrated increased lipid storage in adipocytes under heat stress in this experiment and inhibition of PCK1 activity with 3MPA led to about 20% lower TAG storage under heat stress. This suggests that PCK1 might play an important role in the lipogenic response to high temperature in adipocytes, especially under heat challenge. Increased mRNA and protein level of PCK1 in adipocytes under heat stress demonstrates an increased need for glycerol during hyperthermia in adipocytes. Indeed, heat stress was accompanied by increased mRNA level of AQP7, a gene that is involved in glycerol uptake, confirming this possibility. This agrees with studies that showed induction of AQP7 under heat stress (Sugimoto et al., 2013; Wang et al., 2015). However, AQP7 mRNA level was not affected by PCK1 inhibition.

Expression of genes involved in beta-oxidation (ACOT1 and CPT1) was not affected by heat stress in this study. However, heat stress led to increased mRNA level of PDK4 and LDH. This induction of these genes suggests that heat stress may indeed enhance channeling of substrates into glycolysis and glyceroneogenesis. Pyruvate entry into mitochondrial TCA cycle as acetyl-coA is inhibited by PDK4 (Harris et al., 2002). PDK4 is known as a gatekeeper directing the carbon flux into glycolysis via inhibition of the pyruvate dehydrogenase complex. PDK4 is key to the shift from glycolysis to oxidative phosphorylation (Liu et al., 2017). Lactate dehydrogenase catalyzes the final step in anaerobic glycolysis through the conversion of pyruvate to lactate (Vander Heiden et al., 2009). This step is critical for allowing anaerobic

respiration and glycolysis to continue. The induction of these genes may provide more substrates for PCK1 for glyceroneogenesis in support of increased adipogenesis under heat stress.

A potential mechanism for the increased lipid storage in adipocyte under heat stress might include reduced norepinephrine-stimulated lipolysis. Although basal lipolysis was not different between control cells and those under heat stress, there was a tendency for lower stimulation of free glycerol release in cells under heat stress. The reduction of lipolysis in cells under heat stress may not involve downregulation of ATGL mRNA level because this gene is actually upregulated by heat stress. Although PCK1 inhibition further reduced lipolytic response to norepinephrine in both control and heat stressed cells, and this lipolytic desensitization was accompanied by reduction in the mRNA level of ATGL. This suggests that ATGL might still be involved in some aspects of lipolysis in adipocytes.

Exposure to elevated temperature induces of HSP in vitro which can minimize cellular damage to maintain cellular homeostasis (Cvoro et al., 2004). Among the many HSPs family, HSP70 and HSP90 are well-known members that protects cells and tissues from different stress conditions. Heat shock protein 70 (HSP70) is a molecular chaperone that is involved in protein folding, transportation, and translocation (Cvoro et al., 2004). Accumulation of unfolded or misfolded proteins results in endoplasmic reticulum (ER) stress or unfolded protein response (UPR). Endoplasmic reticulum stress activates markers such as activating transcription factor 4 (ATF4), ATF6, superoxide dismutase 1 (SOD1), x-box binding protein 1 (XBP1) and 78 kDa glucose-regulated protein (GRP78) to restore ER homeostasis (Fulda et al., 2010). The UPR also induces generation of reactive oxygen species (ROS) which could disrupt cellular membrane integrity and function. In this study, we demonstrate that HSP70 is induced by elevated temperature and there was a tendency for HSP90 to be similarly induced. However, PCK1 inhibition with 3MPA reduced HSP70 gene and protein level. The reduction of HSP70 level by 3MPA during heat stress could be linked to the reduced viability of cells in HS that were treated with 3MPA because HSP70 is a recognized survival factor (Silver and Noble, 2012). Additionally, HSP70 could be redirected to the surface lipid droplets in adipocytes during heat stress (Jiang et al., 2007) to prevent misfolding of lipid droplet associated metabolic enzymes and other critical proteins, and translocation of HSP70 to the surface of lipid droplets could potentially lead to

stabilization of lipid droplets during heat stress (Jiang et al., 2007). The reduction of lipolysis in norepinephrine-stimulated cells under high temperature might be a reflection of lipid droplet stabilization by HSP70 during heat stress. Reduction of HSP70 level by 3MPA could contribute to enhanced protein denaturation under HS, and the observed increase in ER stress and cell death.

The increase of ROS concentration, CHOP protein level and the decrease in cell viability in 3MPA treated cells supports the idea that PCK1 might be critical for adipocyte survival and maintenance of cellular homeostasis, especially during heat stress. This may also be linked to decreased HSP70 by 3MPA in HS because HSP70 may be crucial for cellular survival under heat stress induced ER stress. The ER has many important functions, including post-translational modification, folding, and assembly of newly synthesized proteins. Proper ER function is essential for the survival of cells (Araki et al., 2003). We demonstrated that in vitro heat stress promotes oxidative stress and ER stress which is evidenced by intracellular ROS accumulation and increased gene or protein level of ER stress markers such as CHOP, XBP1, ATF4, ATF6, SOD1, and GRP78. Thus, the increase in cellular ROS and ER stress markers like CHOP and the decreased cell viability by 3MPA support a critical role for PCK1 or lipogenesis in alleviating heat stress-induced cytotoxicity.

Heat stress induces increase of cellular ATP concentration for meeting increased cellular need for extra ATP for synthesis of new chaperone proteins such as HSP and others (Soini et al., 2005). Heat adaptation is an energy demanding process that involves interaction of different co-chaperones, co-factors and their substrates and these interactions often involve ATP hydrolysis, which in turn affects substrate turnover rates (Mallouk et al., 1999; Kampinga and Craig, 2010). Although AMP-activated protein kinase (AMPK), is typically activated when intracellular ATP level is low (Mihaylova and Shaw, 2011), the tendency for an increase in AMPK activation in heat stressed cells, despite the high ATP level in these cells, may be to support the increased need for ATP for the adaptive response to heat stress. Thus, AMPK might be a cellular signal for increased energy generation during heat stress in adipocyte.

In summary, inhibiting PCK1 enzymatic activity in vitro decreases the thermotolerance of the adipocyte. Overall, in addition to its well characterized metabolic function in glyceroneogenesis

and lipogenesis, this work indicates a possible implication of PCK1 in heat stress adaptation in pig adipocytes. These findings suggest that upregulation of PCK1 is a critical adaptive feature in pig adipocytes exposed to HS in vitro. Therefore, PCK1 may be an important target for adaptation to elevated temperature in the adipocyte during heat or other stressful conditions such as obesity and type II diabetes treatment

5.6 References

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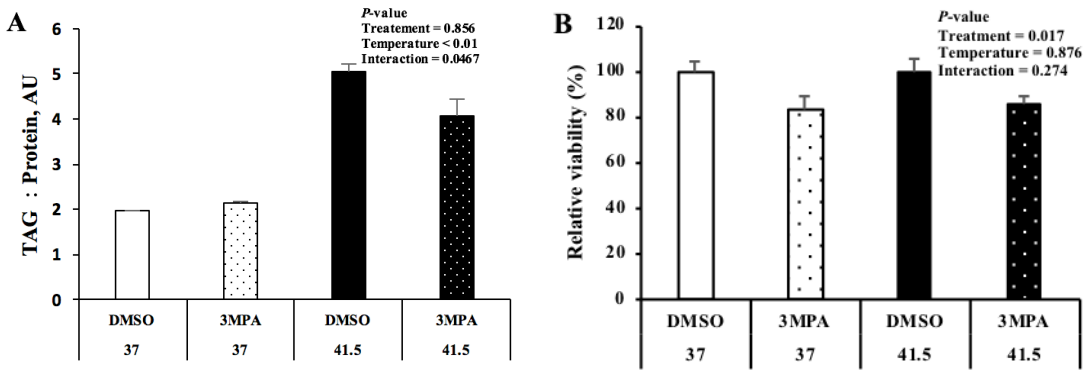
Figure 5.1 Primer sequences for real time-PCR.

Gene	Forward	Reverse
18S	5'-ATC CCT GAG AAG TTC CAG CA-3'	5'-CCT CTT GGT GAG GTC GAT GT-3'
AQP7	5'- AGGCACTTCAGCAGACATCTA -3'	5'- TGGCGTGATCATCTTGGAGG -3'
ACOT1	5'- CCTTTCCTGGGATCGTGGAC -3'	5'- GCAAAACCCTTTCCAGCCAG -3'
CPT1	5'-ATC GTG CCT ATC CTC TGG-3'	5'-CCA GGC CAA GGA GGT TAA-3'
DGAT2	5'-CAC CTA CTC CTT CGG GGA GA-3'	5'-CTT GGA GTA GGG CAT GAG CC-3'
FAS	5'-AGT TTG TGA TGG AGA ACA CGG CCT -3'	5'-TGT TCA CAC GTG GTG CAA GGG TTA -3'
CHOP	5'-AAC AAA GTG GCC ATT CCC CA-3'	5'-ACC ATC CGG TCA ATC AGA GC-3'
XBP1	5'- CGGTGGCCGCTACGTGCACC -3'	5'- GGATATCAGACTCAGAGTCT -3'
SREBP1c	5'- ACC GCT CTT CCA TCA ATG AC -3'	5'- AAT GTA GTC GAT GGC CTT GC -3'
HSP90	5'–GTC GAA AAG GTG GTT GTG TCG–3'	5'- TTT GCT GTC CAG CCG TAT GT-3'
HSP70	5'-TTC GTG GAC AGA AGC CAC AG -3'	5'-TTG CTA GGA TCT CCA CCC GA -3'
PDK4	5'-TGC AAT GAG GGC TAC AGT CG-3'	5'-CGG TCA ATG ATC CTC AGG GG-3'
ATGL	5'TGC CAA TGA GGA CGT GGG3'	5'GCA GCA AGT GAG TGG TTG GT-3'
LPL	5'-ATT CAC CAG AGG GTC ACC TG-3'	5'-AGC CCT TTC TCA AAG GCT TC-3'
PCK1	5'-CCC TGC CTT TGA AAA AGC CC -3'	5'-GGA GAT GAT TTC TCG GCG GT -3'
LDH	5'-ATG TTG CTG GTG TCT CCC TG-3'	5'-TGT GAA CCG CTT TCC AGT GT-3'
SOD1	5'- GTT GGA GAC CTG GGC AAT GT -3'	5'- TCA GAC CAT GGC ATG AGG GA -3'
ATF4	5'AGT CCT TTT CTG CGA GTG GG-3'	5'GGT CGA AGG GGG ACA TCA AG-3'
ATF6	5'GAC CTG TTT TGT CCG TTG TGG-3'	5'ATG TCA CAA GCA AAT GGC CC-3'
GRP78	5'TGAGTGGCTGGAAAGTCACC-3'	5'TTGCCCTGTGCATCTCTTCC-3'

Figure 5.2 Effects of heat stress and PCK1 inhibition with 3MPA on triglycerides (TAG) accumulation and cell viability.



(A) Triglyceride concentration was measured in pig adipocytes under normal conditions (37°C) and heat stress condition (41.5°C) in cells treated from d 7-9 of differentiation with or without 0.05mM of the PCK1 inhibitor (3MPA). (B) Cell viability was measured with MTT assay at the end of d 9 in cells treated or not with 3MPA from d 7-9. Bars represents means ± SE of at least 6 different replicates. Superscript letters (a and b) indicate significant mean difference at $P < 0.05$.

Figure 5.3 Expression of genes related to adipogenesis.

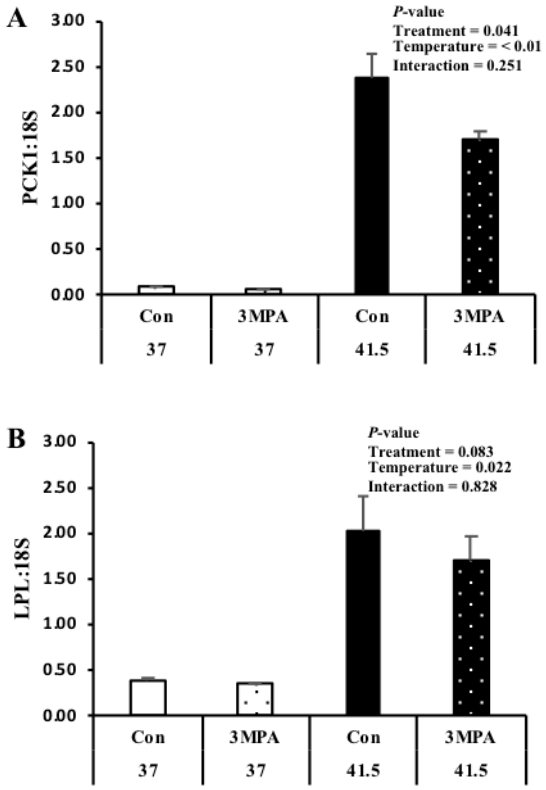
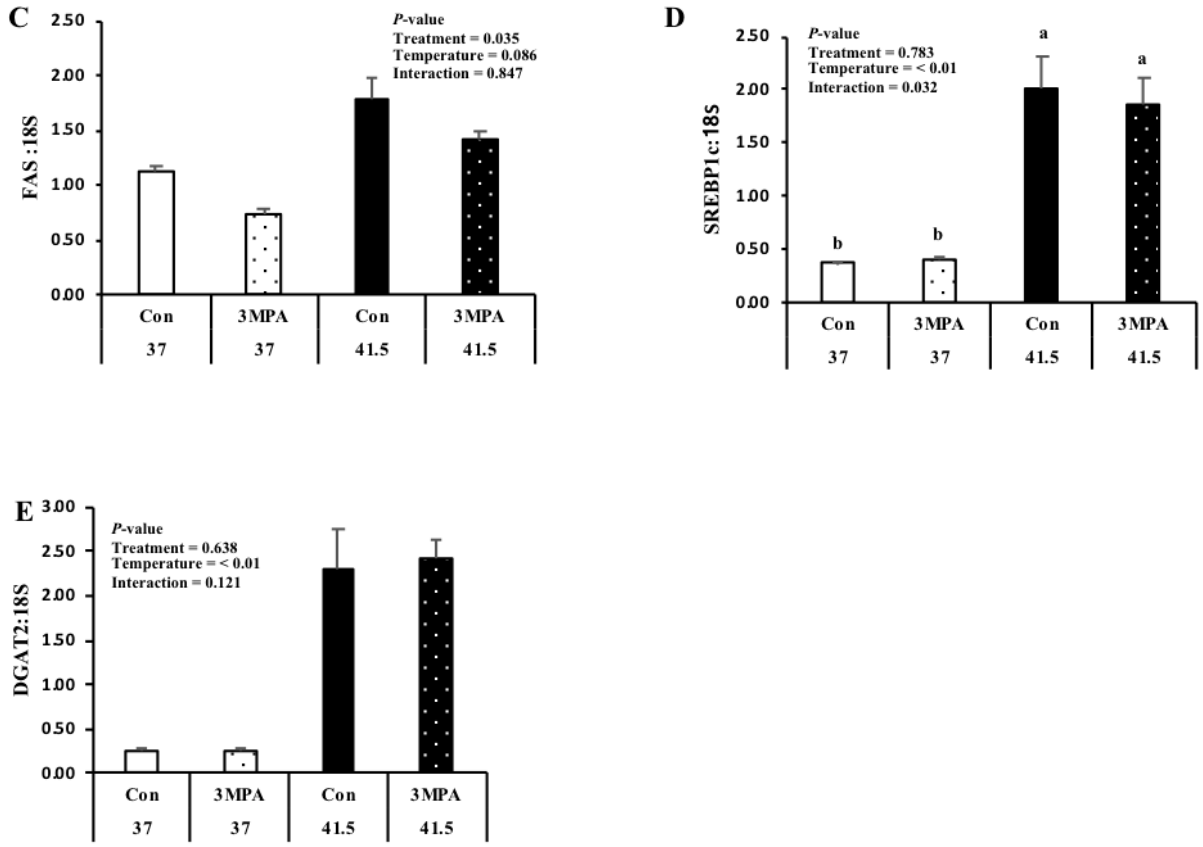
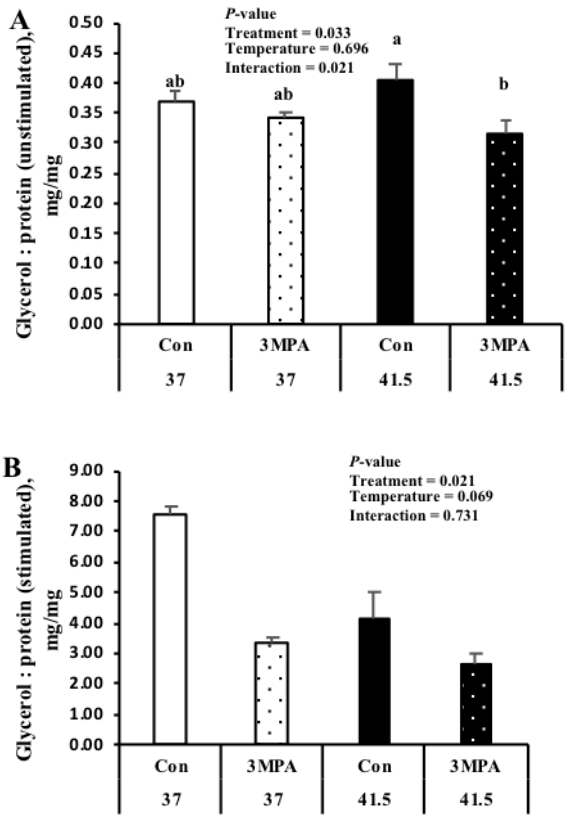


Figure 5.3 Continued



Differentiating adipocytes under normal (37°C) or heat stress (41.5°C) conditions treated from d 7-9 of differentiation with or without 0.05mM of the PCK1 inhibitor (3MPA). (A) PCK1, (B) LPL, (C) FAS, (D) SREBP1c and (E) DGAT2. Bars represents means ± SE of at least 6 different replicates. Superscript letters (a and b) indicate significant mean difference at $P < 0.05$.

Figure 5.4 Basal (unstimulated) (A) or norepinephrine-stimulated lipolysis (B) and mRNA level of ATGL (C) and AQPP7 (D).



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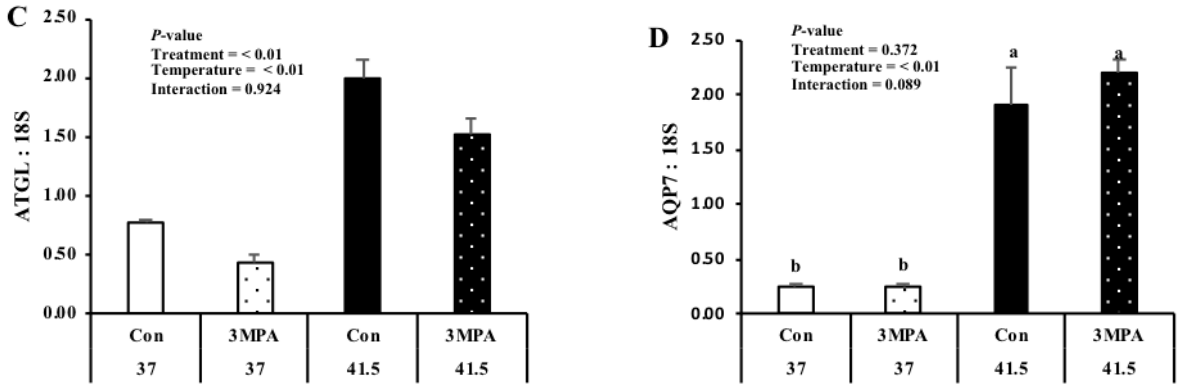
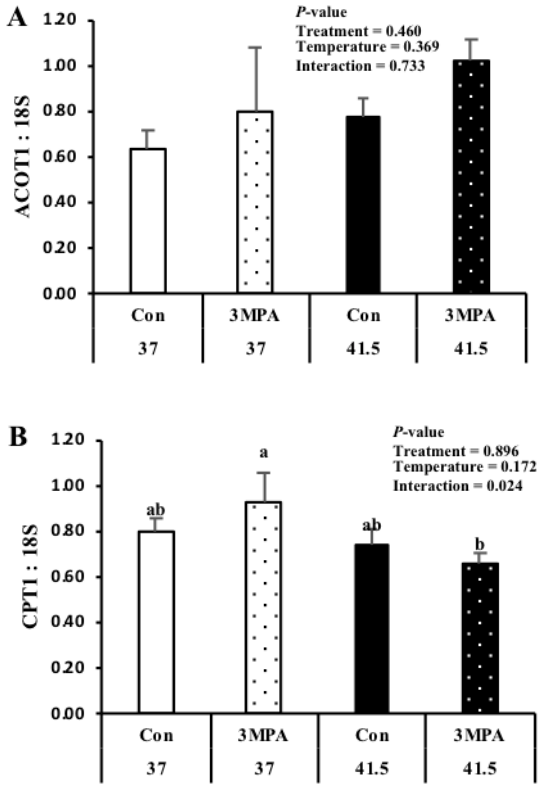


Adipocytes differentiated under normal (37°C) or heat stress (41.5°C) conditions and treated from d 7-9 of differentiation with or without 0.05mM of the PCK1 inhibitor (3MPA). Bars represents means ± SE of at least 6 different replicates. Superscript letters (a and b) indicate significant mean difference at $P < 0.05$.

Figure 5.5 Expression of metabolic markers genes ACOT1 (A), CPT1 (B), PDK4 (C) and LDH(D) and cellular concentration of ATP (nM/µg protein) (E) and p-AMPK/AMPK ratio (F) determined by western blotting.



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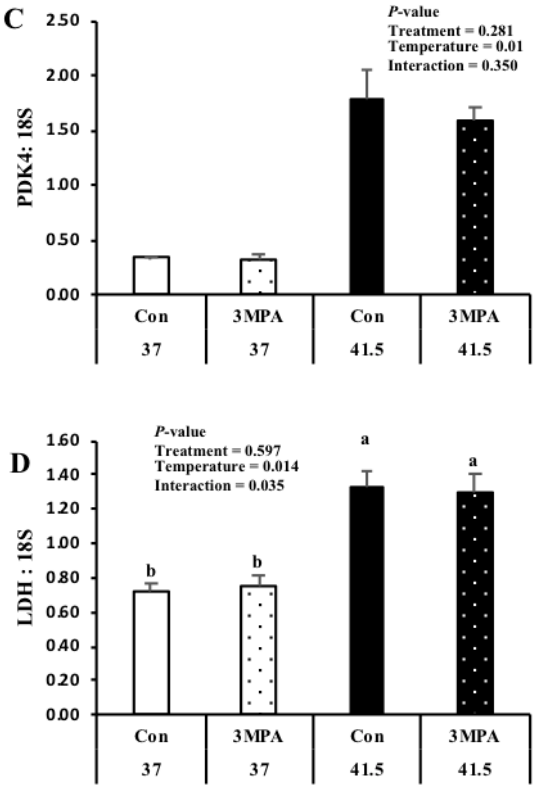

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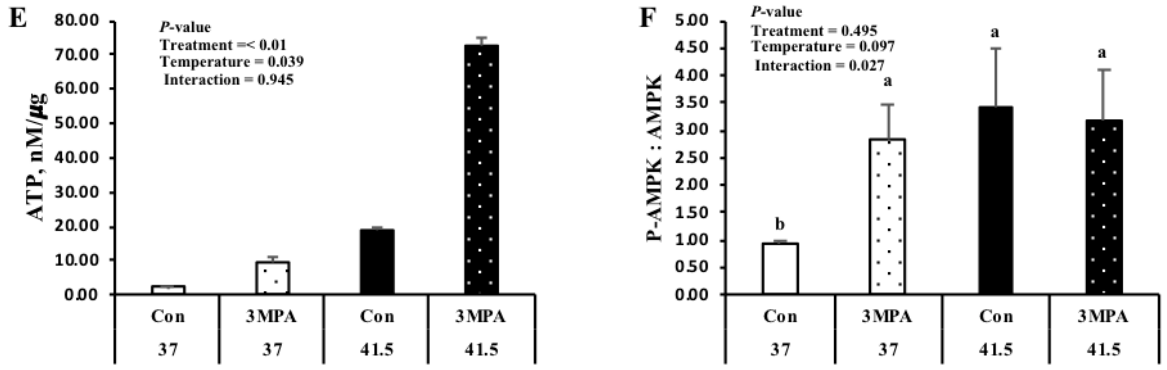
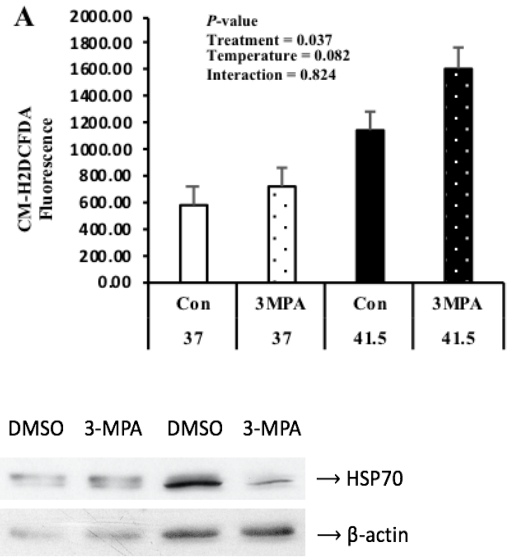
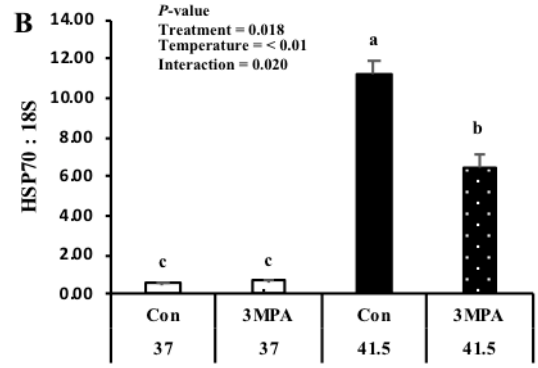
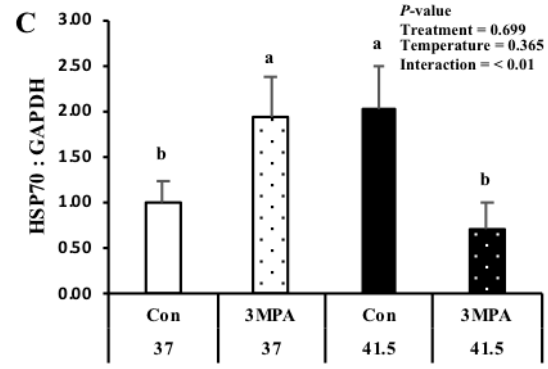


Adipocytes differentiated under normal (37°C) or heat stress (41.5°C) conditions and treated from d 7-9 of differentiation with or without 0.05mM of the PCK1 inhibitor (3MPA). Bars represents means ± SE of at least 6 different replicates. Superscript letters (a and b) indicate significant mean difference at $P < 0.05$.

Figure 5.6 Concentration of reactive oxygen species (ROS) measured by CM-H2DCFDA fluorescence (A), HSP70 mRNA level (B), HSP70 protein abundance (C), HSP90 mRNA level (D), and HSP90 protein abundance (E).







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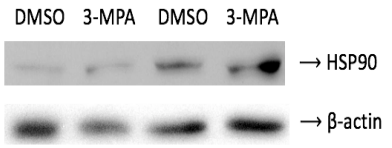
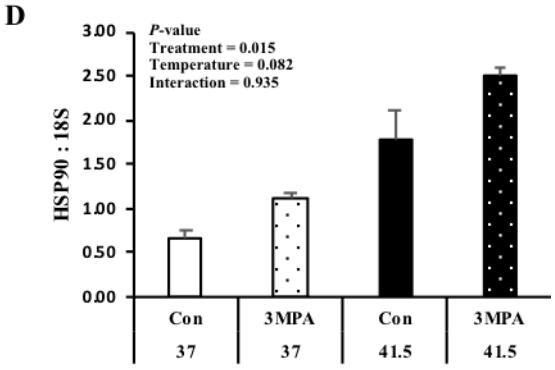
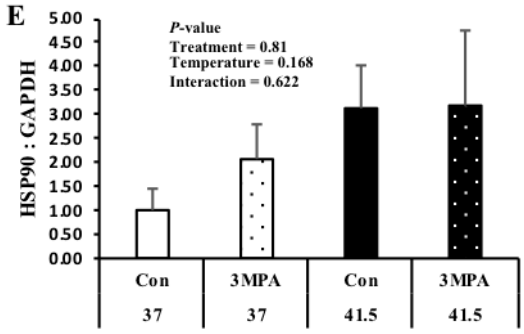






Adipocytes differentiated under normal (37°C) or heat stress (41.5°C) conditions and treated from d 7-9 of differentiation with or without 0.05mM of the PCK1 inhibitor (3MPA). Bars represents means ± SE of at least 6 different replicates. Superscript letters (a, b and c) indicate significant mean difference at $P < 0.05$.

Figure 5.7 Expression of endoplasmic reticulum stress marker genes ATF6 (A), SOD1 (B), ATF4 (C) and protein abundance of EIF2α (D).

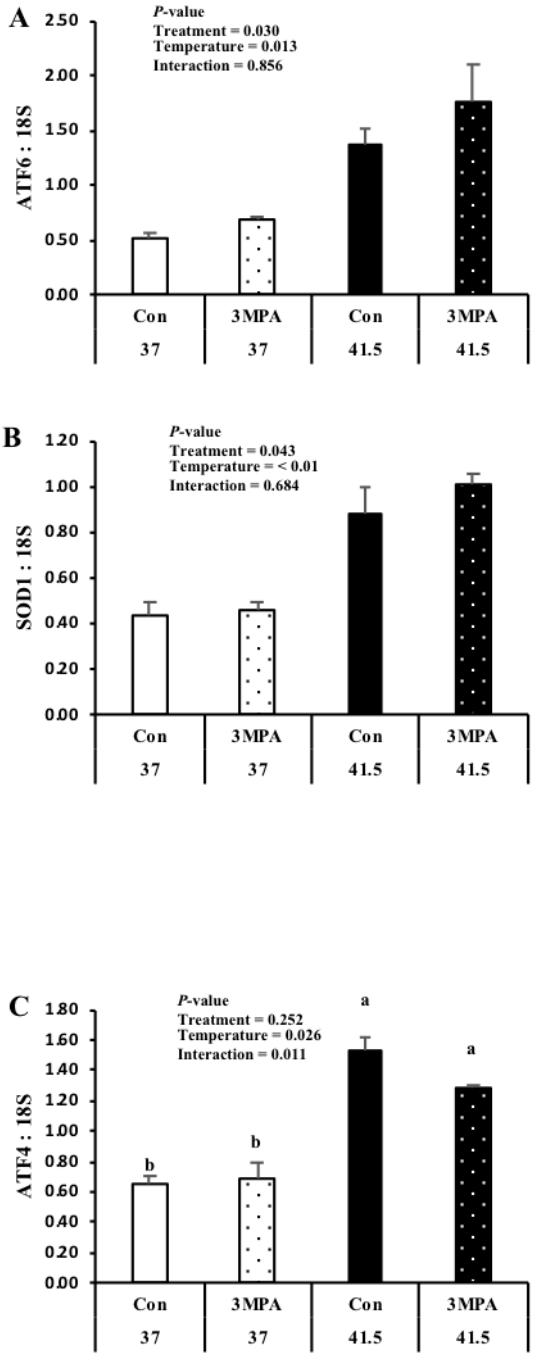
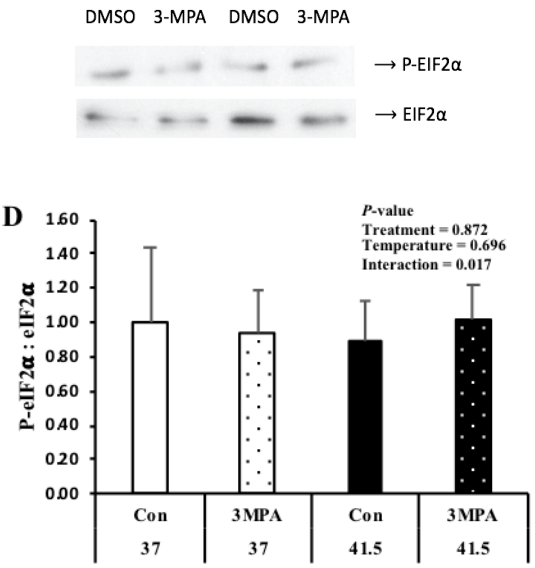


Figure 5.7 Continued



Adipocytes differentiated under normal (37°C) or heat stress (41.5°C) conditions and treated from d 7-9 of differentiation with or without 0.05mM of the PCK1 inhibitor (3MPA). Bars represents means ± SE of at least 6 different replicates. Superscript letters (a and b) indicate significant mean difference at $P < 0.05$.

Figure 5.8 Expression of endoplasmic reticulum stress markers.

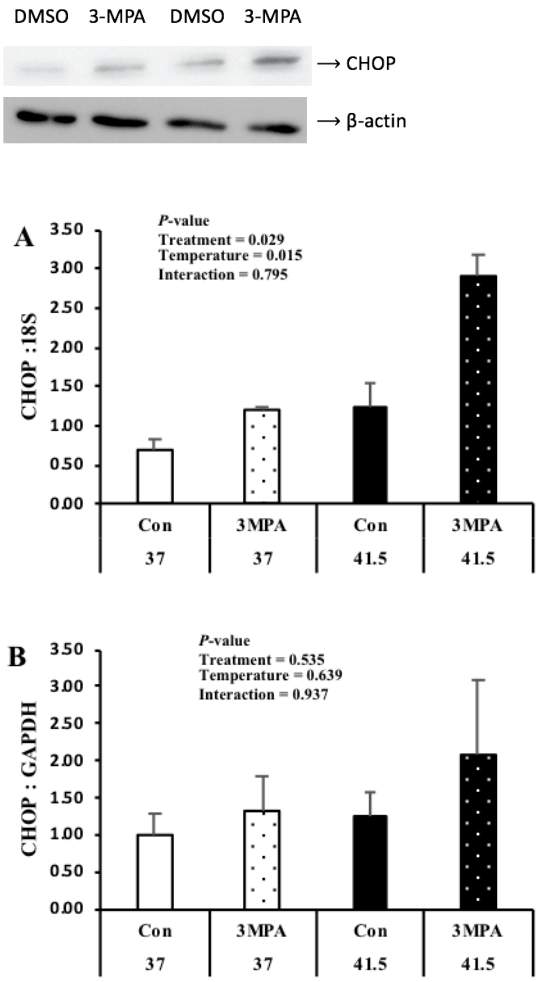
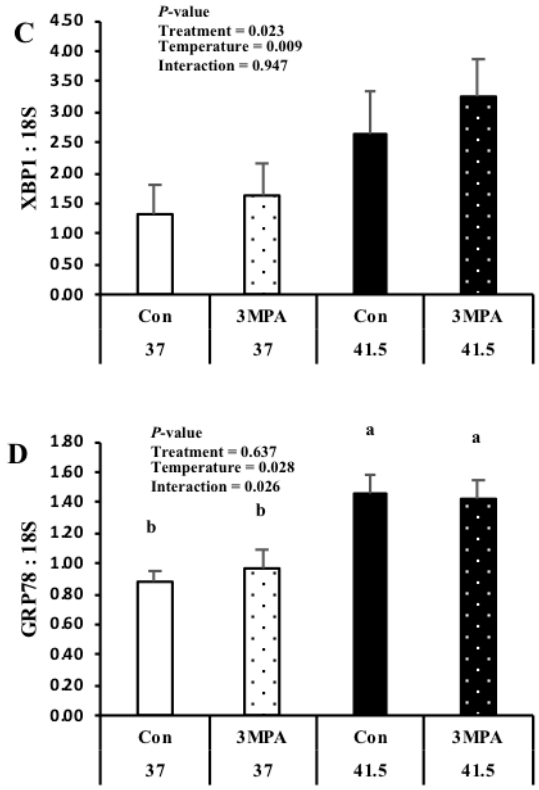


Figure 5.8 Continued



CHOP mRNA (A), CHOP protein (B), XBP1 mRNA (C) and GRP78 mRNA (D) in adipocytes differentiated under normal (37°C) or heat stress (41.5°C) conditions and treated from d 7-9 of differentiation with or without 0.05mM of the PCK1 inhibitor (3MPA). Bars represents means ± SE of at least 6 different replicates. Superscript letters (a and b) indicate significant mean difference at $P < 0.05$.

CHAPTER 6. METABOLOMICS OF HEAT STRESS RESPONSE IN PIG ADIPOSE TISSUE REVEALS ALTERATION OF PHOSPHOLIPID AND FATTY ACID COMPOSITION DURING HEAT STRESS.

6.1 Abstract

To determine the effect of heat stress (HS) on adipose tissue metabolome, a combination of liquid chromatography-mass spectrometry (LC-MS) based metabolomics profiling approaches was applied to characterize changes of metabolite classes in adipocytes differentiated in culture (in vitro) and mesenteric adipose tissue of pigs exposed to high temperature (in vivo) and mesenteric adipose tissue of pigs exposed to HS (in vivo). Effect of HS on the composition of individual fatty acids in cultured adipocytes, mesenteric adipose tissue and serum of animals was also investigated using gas chromatography (GC) analysis. In vitro, preadipocytes were differentiated either under control (37 °C) or elevated temperature (HS, 41.5 °C) temperature for 9 d. For the animal experiment, pigs were kept either in control (Con) environment (20 °C) with ad libitum feed intake, heat stress (35 °C) temperature with ad libitum feed intake (HS) or at 20 °C with pair feeding to the HS pigs. In cultured cells, heat stress increased triglyceride and decreased monoacylglycerol ($P < 0.05$) species accumulation compared with control. Phosphatidylinositol and phosphatidylserine concentrations were increased by HS whereas phosphatidylcholine, phosphatidylethanolamine and phosphatidylglycerol were decreased relative to control ($P < 0.05$). Heat stressed adipocytes in culture also had higher concentrations of saturated and monounsaturated fatty acids ($P < 0.05$) relative to control. Pathways of proline and biotin metabolism were elevated ($P < 0.05$) by heat stress in adipocytes. The metabolomics signatures in adipocytes cultured under HS indicates that pathways centered around diacylglycerol (DAG) metabolism are impacted by HS. In adipose tissue from animals under heat stress, there was increased ($P < 0.05$) abundance of 4,8 dimethylnonanoyl carnitine ($P < 0.05$). Heat stressed animals also had higher (P<005) serum linoleic, total polyunsaturated fatty acids (PUFA) and decreased total saturated fatty acid than PF ($P < 0.05$). These results indicate that HS elevates lipogenic pathways while suppressing fatty acid oxidation and demonstrate the usefulness of metabolomics analysis as a tool for determining the impact of HS in pig tissues.

Key words: adipocyte; heat stress; lipids; metabolomics; lipidomics

6.2 Introduction

Heat stress is a major environmental factor against optimal production efficiency in swine. An important adaptive response to heat stress (HS) is the induction of heat shock proteins (HSP) which help in heat adaptation (Feder and Hofmann, 1999). However, HS response also involves other mechanisms, such as alteration in lipid metabolism (Schmidt and Widdowson, 1967; Katsumata et al., 1990). This is because lipids play a very important role in regulation of several biological processes involved HS response. Therefore, determining the impact of HS on lipid metabolites is an important step towards a better understanding of the role of different lipids in heat stress adaptation.

It has been demonstrated that HS increases lipid retention by decreasing lipolytic rates or increasing adipose tissue lipoprotein lipase in rodents (Schmidt and Widdowson, 1967; Katsumata et al., 1990), pigs (Heath, 1983; Christon, 1988) and chickens (Geraert et al., 1996). Additionally, in our earlier work (Qu et al., 2015), HS stimulated cell autonomous increase in lipogenesis and triglyceride storage in pig adipocytes (Qu et al., 2015). Therefore, changes in cellular and tissue lipid composition could indicate that lipids are an integral part of physiological and metabolic adaption to heat stress. In fact, it has been suggested that membrane lipid physical state could control heat sensing and signaling (Escriba et al., 2008).

Qu et al., (2016) showed that heat stress resulted in increased gene and protein level of heat shock protein 70 (HSP70), CCAT/enhancer-binding homologous protein (CHOP) in the mesenteric fat compared with animals kept in thermoneutral environment. In addition, heat stress led to increased gene and protein level of phosphoenol pyruvate carboxykinase (PCK1), suggesting that heat stress might lead to tissue stress and elevated glyceroneogenesis in adipose tissue. There was also a modest increase in blood urea nitrogen in male pigs in heat stress compared with control animals (Qu et al., 2016). However, these indicators did not provide a comprehensive insight into tissue responses to heat stress that could increase understanding of effect of heat stress in pigs. Therefore, in this study, we have conducted further metabolomics analysis of mesenteric adipose tissue and plasma of animals from Qu et al. (2016) for a better

understanding of the extent of alteration of lipid species and other metabolites by heat stress. These metabolites and lipids species may serve as useful biomarkers associated with heat stress response. Determining the identities of these metabolites may open a window for investigating the mechanism of heat stress sensing, signaling, and adaptation, and may provide information that can be utilized for achieving efficient animal production. Therefore, the objective of this experiment was to determine, using metabolomics analysis, a comprehensive impact of HS on lipid and non-lipid metabolites in adipocytes that were differentiated in vitro and mesenteric adipose tissue of pigs.

6.3 Materials and methods

Experiment 1: Effects of heat stress in pigs.

Purdue Animal Care and Use Committee approved all animal protocols described in this publication. Details of animal experimentation, tissue collection and handling have been described in Qu et al. (2016). Ossabaw pigs (female n=15, male n=15) at 6 months of age were randomly allocated to three treatments (female n=5 and male n=5 per treatment): control (Con) group with room temperature at 20 °C ± 1 °C and relative humidity 53% to 78%, and had ad libitum feed intake; pair-fed (PF) treatment with same room temperature and relative humidity as Con group, and pair-fed to the feed intake of heat stress (HS) pigs; HS group with room temperature at 35 °C ± 1 °C and relative humidity 45% to 65% with ad libitum feed intake. Pigs were penned individually and were allowed to adjust to their environment for 7 d before treatments were applied for another 7 d. Pigs in the pair-feeding treatment were fed twice daily at 0900 and 0500 h with a quantity of feed equal to the previous day's FI of pigs in the HS treatment. The feed met or exceeded the nutrient requirements of pigs of that age (NRC, 2012) (Table 6.1).

On d 7, pigs were euthanized as described (Qu et al., 2016) and mesenteric adipose tissues collected from areas flanking the mid-jejunum were flash frozen in liquid nitrogen before storage in -80°C freezer. On d7 of experiment, pigs were euthanized following sedation with intramuscular injection of atropine, tiletamine-zolazepam and xylazine, and asphyxiated with CO2, followed by pneumothorax and cardiectomy while under anesthesia. Mesenteric adipose

tissues were collected from areas flanking the mid-jejunum and flash frozen in liquid nitrogen before storage in -80°C freezer. Blood was collected into vacutainer tubes by jugular venipuncture before feeding on d7 of the experiment. Serum was recovered from whole blood following centrifugation at 10,000 ×g for 10 min at 4°C.

Experiment 2: Cell isolation and in vitro culture

Isolation and culture of primary porcine preadipocytes were based on the protocol described previously (Ramsay, 2005; Qu et al., 2015). Briefly, preadipocytes (SVC) were isolated from the inner layer of subcutaneous adipose tissue from male juvenile piglets (< 7 d old). Tissue was kept in buffered saline (0.15 *M* NaCl, 10 m*M* HEPES, pH 7.4) at 37^{o}C and cut into small pieces with a pair of sterile scissors followed by incubation in a digestion cocktail (10 m*M* $NaHCO_3$, 10 m*M* HEPES, 5 m*M* D-glucose, 120 m*M* NaCl, 4.6 m*M* KCl, 1.25 m*M* $CaCl_2$, 1.20 m*M* $MgSO_4$, 1.20 m*M* KH_2PO_4, 3% BSA) containing collagenase (Collagenase type I, 1mg/mL, Worthington Biochemical Corp., Lakewood, NJ) in a shaking water bath at 37^{o}C for 45 min at 120 oscillation/min. Adipocytes were separated from SVC pellet by centrifugation at 2,000 × g for 10 min at 4°C. The SVC was washed twice in the digestion cocktail. The SVC pellet was resuspended in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) medium (Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum (Mediatech, Manassas, VA), 1% antibiotic-antimycotic (Sigma Aldrich, St. Louis, MO) and incubated in a humidified incubator with 5% CO_2 and 95% air. Cells were differentiated under normal (37°C) or HS (41.5°C) temperature environments for 9 d in a differentiation medium: DMEM/F12 with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic, 1μ*M* insulin, 1μ*M* dexamethasone (1m*M*), 1μ*M* rosiglitazone, 1 μ*M* biotin, 1μ*M* triiodothyronine (T3), 1 μ*M* pantothenic acid after reaching confluence. Medium was replaced every 3 d and rosiglitazone was removed from the differentiation media after d 3 of differentiation. Cells were fully lipid-filled by d 9.

Sample preparation for metabolomics analysis

Adipocytes from the in vitro culture experiment at either 37°C or 41.5°C culture conditions as described above were scraped into microcentrifuge tubes and homogenized in 4 ml/gram of cold methanol and 0.85 ml/gram cold water. Only mesenteric adipose tissues from HS and PF pigs

were compared. Frozen mesenteric adipose tissues were thawed on ice and also homogenized in cold methanol as described for in adipocytes from the vitro culture. Both adipocyte and mesenteric adipose tissue samples were thereafter processed similarly. Cold chloroform (4 ml/g) and 2 ml/gram cold water were added. Samples were then vortexed for 60 sec and kept on ice for 10 min to allow phase separation and thereafter centrifuged for 10 min at 10,000 × g in 4 ºC to remove precipitated proteins and tissue debris. The upper polar phase was transferred into new Eppendorf tube for metabolomics analysis of polar compounds and the lower non-polar organic (lipids) phase into a new tube for metabolomics (lipidomics) analyses (Wu et al., 2008).

Non-targeted metabolomics analysis of polar and non-polar (lipids) compounds

The analysis was performed on an Agilent 1100 series HPLC system coupled to an Agilent MSD-TOF (time-of-flight) MS (Agilent Technologies, Santa Clara, CA, USA). The compounds were separated using a Waters Atlantis T3 column (150 mm x 2.1 mm, 3-µm particle size) in a 50-min run at 30°C with an injection volume of 5 µl. A gradient extraction procedure was applied using a binary mobile phase composed of 0.1% formic acid (v/v) in double-distilled water (eluent A) and 0.1% formic acid (v/v) in acetonitrile (eluent B) at a flow rate of 0.3 ml/min. The following linear gradient program was applied: 0 min, 0% B; 1 min, 0% B; 39 min, 80% B; 40 min, 0% B; 50 min, 0% B. The MS was equipped with an electrospray ionization (EIS) source. The data generated were collected with a mass-to-charge ratio (m/z) in the range of 75 to 1000 with a 1.4 spectra/s acquisition rate. The operating conditions for ESI were as follows: nebulizer gas (N2) pressure, 35 psi; drying gas (N2) flow, 9.0 L/min; and drying gas temperature, 350°C. The capillary and fragmentor voltages were set at 3500 and 135 V, respectively. Accurate mass calibration was carried out by the continuous infusion of Agilent Reference Mass Solution (reference number G1969-85001) using the following reference masses: 121.0509 and 922.0098 m/z.

Non-targeted metabolomics analysis of non-polar organic compounds (lipids) was performed on the same Agilent 1100 system using a Waters Xterra MS C18 column (5 µm, 150 x 2.1 mm i.d) with an injection volume of 10 uL. A binary mobile phase consisting of solvent systems A and B were used in gradient elution where A was water, 10 mM ammonium acetate, 0.1% formic

acid (v/v) and B was 50% acetonitrile, 50% isopropyl alcohol, 10 mM ammonium acetate, 0.1% formic acid (v/v). The mobile phase flow rate was 0.3 mL/min. Initial conditions were set at 65:35 A: B and held for 1 min, followed by a linear gradient to 20:80 from 1 to 10 min, followed by a linear gradient to 0:100 from 10 to 20 min and held at 0:100 for 12 min. Gradient conditions were re-equilibrated to 65:35 A: B from 32 to 33 min and held for 7 minutes at initial conditions prior to the next run. Following separation, the column effluent was introduced by positive or negative mode electrospray ionization (ESI) into an Agilent MSD-TOF spectrometer. ESI capillary voltage was 3.5 kV, nebulizer gas pressure was set at 35 psi, gas temperature was 350 °C, drying gas flow rate was 9.0 L/min, fragmentor voltage was set to 135 V, skimmer 60 V and OCT RF V 250 V. Mass data (m/z 100 - 1200) were acquired and analyzed using Agilent Mass Hunter software. This mass data will lead to identification of precursor ion masses and specific compound names whose relative abundance is given by the ratio of their peak area in the chromatogram generated compared to the control sample. Relative abundances of compounds were given as fold changes relative to the control based on the area of the chromatographs. Accurate mass determination was ensured by the continuous infusion of Agilent Reference Mass Solution (G1969-85001).

GC Analysis of Fatty Acid Methyl Esters

Total lipids from in vitro differentiated adipocytes, serum, and mesenteric adipose tissue were extracted based on the modified method of (Folch et al., 1957). Samples were extracted in a 2:1 (vol/vol) chloroform: methanol organic solvent mixture. Fatty acid methyl esters (FAME) were prepared in a reaction with sodium methoxide in methanol. Then FAME was dissolved in hexane for GC analysis. The FAME were quantified using an Agilent 7890 GC equipped with a FID detector and also Agilent 5975 MSD detector (Agilent, Santa Clara, USA). The GC/FID was equipped with a 60 m DB-5 capillary column (0.25 mm ID, 0.25 μm film thickness; J&W Scientific). The FAME were identified by retention time and fragmentation pattern and quantified by peak area against a standard (Supelco 37 component FAME mix; Sigma-Aldrich, St. Louis, MO). GC/MSD analysis using a rt-2560 capillary column (100 m × 0.25 mm internal diameter; 0.2 μm film thickness; Restek, Bellefonte, PA, USA). The GC/FID detector temperature program was set to 90 ºC to 180 ºC at 10 ºC /min, then to 285 ºC at 3 ºC /min;

injector temperature 200 ºC, detector temperature 290 ºC. Total run time 47 min. GC/MSD detector temperature program was set to 50 ºC for 5 mins to 125 ºC at 10 ºC /min, then to 250 ºC at 4 ºC /min hold 12.25 min; injector temperature 250 ºC, detector temperature 300 ºC. Total run time 56 min. Results were presented and analyzed as a molar percentage of total FAME.

Data processing and statistical analysis

Agilent's Mass Hunter Qualitative Analysis software (v. B.06) was used to identify individual chromatographic peaks. The molecular feature extraction algorithm was used as a first pass, followed by the find by ion algorithm. Agilent's Mass Profiler Professional software (v. 12.6.1) were used to identify statistically significant biomarkers by performing paired t-test. Peaks were aligned across all samples in a data set to account for instrumental drifts in retention time and mass. Each compound was normalized first by external scalar (to account for sample weights), and then Percentile Shift (using 75% option). Compounds were baselined, then Filtered on Flags, retaining only compounds that appeared in 2 or more samples. Compounds were then Filtered by Frequency, keeping only compounds that appeared in a defined percentage of samples in at least one treatment type. A PCA plot was generated, which a quality control step to identify possible outliers. A t-test was performed to compare treatment differences. A fold change criterion (2 for lipidomic analysis and 25 for metabolomics analysis) was employed whereby samples from heat stress treatments were compared to control and these ratios statistically analyzed. Statistically significant compounds determined by t-test analysis between control and heat stress treatments were queried on the Human Metabolome Database (HMDB) and the MetaCore software (Encinitas, CA) was used for pathway analysis. A peak must be present in 4 out of 6 samples to determine presence/absence. Comparisons to assess the significance of differences in fatty acid profiles in adipocyte were performed using one-way ANOVA with a Tukey HSD posthoc test. Differences in tissues and sera were analyzed with two-way ANOVA followed with the Tukey comparison test.

6.4 Results

Animal Performance in response to heat stress

Performance responses of animals in response to treatment has been previously described in detail (Qu et al., 2016). Briefly, pigs in the HS treatment had approximately 40% lower FI in comparison to the CON treatment after 7 days of treatment ($P < 0.01$; Qu et al., 2016). However, as designed, the FI of the PF pigs was similar to the of the HS pigs. There were no differences in final BW and daily gain in all treatments. The HS pigs had elevated skin temperature (+6°C) and a 2-fold increase in respiration rate compared with CON and PF pigs ($P < 0.01$; Qu et al., 2016). Final rectal temperature was +0.3°C higher ($P < 0.05$; Qu et al., 2016) in HS pigs than CON and PF. There was a tendency for a higher ($P < 0.06$) protein level of HSP70 in the mesenteric adipose tissue of HS pigs compared to CON (2 fold) and PF (1.5 fold) pigs. There was also a significant increase ($P < 0.01$; Qu et al., 2016) in the mRNA level of phosphoenolpyruvate carboxykinase 1(PCK1) in the mesenteric adipose tissue of HS pigs compared to CON (10.7 fold) and PF (4 fold). Plasma metabolites such as triacylglycerol (TAG), free fatty acids (FFA) and glucose were not affected by treatment. However, males in HS had elevated (1.4 fold, $P<0.05$) blood urea nitrogen (BUN) relative to males in the CON treatment.

Effects of heat stress on adipocyte lipidome (non-polar compounds)

A total of 3494 ionic species were detected using both positive and negative ionizations in a non-targeted approach. Multivariate analyses were used to explore the differences between locations. Due to the similarities in the patterns of results from both negative and positive ionizations by principal component analysis (PCA), only the positive ionization results are shown. As the PCA plots of non-polar compounds show (Figure 6.1), samples were separated based on temperature. The heat map shows that the abundance of several lipids was different between CON and HS treatments (Figure 6.6). The whole lipidome reveals that 718 lipid species were significantly different between control and heat stressed adipocytes. The lipid species that were identified were further separated by their classes and expressed based on whether their total count was

increased or decreased in heat stress relative to control. Among them were glycerophospholipids, glycerolipids, sphingolipids, steroids and steroid derivatives, prenol lipids, linoleic acids and derivatives and fatty acyls (Figure 6.2A). Among the glycerophospholipid species, glycerophosphoserines, glycerophosphoglycerols, glycerophosphoethanolamines, glycerophosphoinositols, glycerophosphoglycerophosphates and glycerophosphates were identified (Figure 6.2B). Phosphatidylcholine and phosphatidylethanolamine were the two most affected glycerophospholipids.

Glycerolipids were classified to triglycerides, diacylglycerols, and monoacylglycerols (Figure 6.2C). Triglycerides were the most abundant in the heat stress group, and monoacylglycerols were most abundant in the control group. A comprehensive list of lipid metabolites is presented in supplementary Table 6.1.

Effects of heat stress on the metabolome of polar compounds in adipocytes

The metabolome of polar compounds also separated by temperature using PCA (Figure 6.3A). The heat map showed two distinct patterns of regulation of the compounds and indicates that the abundance of several compounds was inversely regulated in heat stress and control (Figure 6.7). Additionally, metabolomics pathway analysis (Figure 6.3B) revealed upregulation of several metabolic pathways including proline, dGTP, biotin, insulin, galactose, acetyl-coA and transcription factor carbohydrate response element binding protein (ChREBP) metabolism by heat stress.

Effects of heat stress on mesenteric adipose tissue lipidome (non-polar compounds)

The PCA plot of mesenteric fat nonpolar compounds indicates sex effects in the separation of metabolites such that the metabolites separated better by temperature better in the boars (Figure 6.4A) than gilts (Figure 6.4B). There was also a higher ($P < 0.05$) amount of PE (18:0/22:1(13Z) and PC (16:1(9Z)/20:0) in boars in HS than PF (Figure 6.4C).

Effects of heat stress on the metabolome of polar compounds in the mesenteric adipose tissue

The PCA plot of polar compounds reveal a clear separation between the PF and HS treatments in the boars (Figure 6.5A) and gilts (Figure 6.5B). Metabolic pathway analysis revealed increased ($P < 0.05$) metabolism of arginine, adenine, 4,8 dimethylnonanoyl carnitine in gilts in HS than PF (Figure 6.5C).

Fatty acid profiles of adipocyte, serum and mesenteric adipose tissue

Adipocyte fatty acid profile is presented in Table 6.2. Adipocytes in exposed to high temperature had a greater proportion of total saturated fatty acids (SFA) than Con, and higher ($P < 0.05$) composition of myristic (C14:0) and palmitic (C16:0) acids than any other single fatty acid (9.57% and 30.52% increase respectively). Adipocytes in HS also had a lower ($P < 0.05$) proportion (21.81% decrease) of mono-unsaturated fatty acids (MUFA) and lower MUFA: SFA ratio than Con. The composition of the MUFA palmitoleic (C16:1) was lower ($P < 0.001$) in the HS group than Con (57.30% decrease). Additionally, linoleic (C18:2n6c) and eicosapentaenoic acid (EPA, 20:5n3), both PUFA were lower ($P < 0.05$) in HS treatment (39.43% and 15.00% decreases, respectively) than Con.

Serum fatty acid profile is presented in Table 6.3. Concentration of stearic acid (C18:0) was decreased in HS (17.32% decrease) compared to PF ($P < 0.002$). However, there was an increase in linoleic acid in the serum of HS pigs compared to PF (43.91% increase) ($P < 0.004$). There was a decrease ($P < 0.003$) in total saturated fatty acid (SFA) in HS relative to PF (8.6% decrease). However, total polyunsaturated fatty acid (PUFA) increased 33.38% ($P < 005$) in HS pigs relative to PF. Therefore, the PUFA/SFA ratio was higher ($P < 0.003$) in HS than PF and control. Fatty acid analysis of mesenteric adipose tissue is presented in Table 4. There was no effect of treatment on the overall fatty acid profile, although gilts had a lower ($P < 0.002$) level of linoleic acid, higher ($P < 0.03$) level of gadoleic acid and a lower PUFA/SFA ratio ($P < 0.028$) than boars.

6.5 Discussion

Previous studies have shown that HS causes increased lipid retention in rodents (Katsumata et al., 1990), pigs (Christon, 1988, Kouba et al., 2001) and chickens (Geraert et al., 1996). In Qu et al., (2015), it was also demonstrated that exposure to high temperature increased de novo lipogenesis and triglyceride storage in pig adipocytes. Thus, there was a need to get a better understanding of effects of HS on lipid metabolism and metabolite changes in pigs. In contrast, other studies showed that HS resulted in leaner carcasses in pigs (Cruzen et al., 2015; Johnson et al., 2015), and studies by Sanz-Fernandez et al. (2015) did not find any significant effect of HS on markers of insulin sensitivity in adipose tissue in pigs.

Differences in measures of adiposity or adipose-specific responses to HS from different investigators likely reflect effects of dissimilar experimental conditions such as duration and intensity of HS and whether heat stressed pigs were being compared to ad libitum fed pigs kept in thermoneutral temperature or with those that were pair-fed to heat stressed pigs, as we have done in this study, to account for potential effects of feed intake differences. In any case, determining comprehensive changes in lipid and other metabolite classes are needed to elucidate mechanism of HS response in pigs. Such an understanding could be useful for developing better management strategies for dealing with the negative consequences of HS in pigs.

Pig adipocytes grown in culture respond to HS with increased triglyceride storage (Qu et al., 2015). Our previous work in pigs exposed to HS has also shown that HS in pigs is accompanied by adipose tissue-specific responses that favor increased triglyceride storage due to increased gene and protein level of PCK1, a lipogenic enzyme (Qu et al., 2016). This observation is confirmed in the metabolomics analysis and breakdown of glycerolipids conducted in this study, especially from the in vitro differentiated adipocytes. The reduction in monoacylglycerols and diacylglycerols and the upregulated triacylglycerol species (Figure 2C) in HS adipocytes support a greater accumulation of triacylglycerols in adipocytes during heat stress at the expense of monoacylglycerols and diacylglycerols. In parallel, we also found increased metabolism of biotin and acetyl-coA metabolic pathways. Biotin is a co-factor that is used by the enzyme acetyl-coA carboxylase for the synthesis of malonyl-coA, in the first committed step of fatty acid synthesis (Chang et al., 1967). Thus, the metabolomics analysis suggests increased diversion of glucose to

fatty acid and triacylglycerol synthesis during HS. This may be due to reduction of glucose oxidation during heat stress in adipocytes exposed to HS in vitro.

The default fatty acid from activities of acetyl-CoA carboxylase and fatty acid synthase (FAS) is palmitic acid (Smith, 1994). In support of increased de novo fatty acid synthesis, concentration of palmitate abundance was increased in adipocytes in HS in vitro (Table 2). This result is consistent with our previous finding that heat stress leads to increased FAS expression in pig adipocytes grown in culture (Qu et al., 2015). In addition, HS leads to increased concentration of palmitoleic acid (C16:1) in heat stressed adipocytes (Table 2). This raises the possibility of increased activity of steroyl-CoA desaturase (SCD1) for the desaturation of palmitic acid to palmitoleic acid (Enoch et al., 1976) during HS. Increased palmitic acid concentration in adipocytes under HS indicates a potential role of this fatty acid in the regulation of cellular membrane fluidity as part of the mechanism of homeoviscous adaptation to HS (Ernst et al., 2016). It is known that HS induces fluidization of cellular membranes (Vigh et al., 1998; Leach and Cowen, 2014). Because fluidity of the cell membrane is important for membrane function, cellular membrane fluidity is maintained within a narrow range through homeoviscous adaptation to maintain cellular function under HS (Dymond, 2015). Thus, increased synthesis of palmitate, a saturated fatty acid, under HS may suggest its increased use to counter the increased membrane fluidity caused by HS. Indeed, the abundance of phosphatidylcholine species with two palmitic acids (PC (16:0/16:0)) was increased in heat stressed in vitro differentiated adipocytes. The most abundant membrane phospholipid is phosphatidylcholine (van Meer et al., 2008). Thus, increasing the abundance of this saturated phosphatidylcholine might be an adaptive mechanism to correct the fluidization of adipocyte membrane by HS. This is presumably through an increase the concentration of phosphatidylcholine species with saturated fatty acids which are expected to decrease membrane fluidity.

Pathway analysis of in vitro cultured adipocytes under heat stress also show enhanced proline metabolism. Metabolism of proline to 4-hydroxyproline is essential for the thermal stability of the collagen triple helix. Collagen that lacks hydroxyproline residues has a lower stability and melting temperature (Kubow et al., 2015). Although heat stress increases triglyceride synthesis, it causes membrane fluidity. Thus, increased production of collagen with 4-hydroxyproline

might be an attempt to increase cellular rigidity to counter the fluidization that is brought about by heat stress. Therefore, regulation of extracellular matrix structure might be part of the adaptive response to heat stress in the adipocyte. On the other hand, proline has a potential a role to suppress reactive oxygen species (ROS) and apoptosis in mammalian cells (Krishnan et al., 2008) and increased proline metabolic pathway might direct proline to this anti-ROS and anti-apoptotic functions as well.

In the animal experiment, we chose a pair-feeding strategy to allow separation of observed effects that were due to differences in feed intake between animals in control environment versus those in an elevated temperature. Indeed, this need informed our choice of the PF and HS serum and mesenteric adipose tissue samples for the metabolomics analyses. Thus, responses obtained were independent of feed intake reduction. Mesenteric adipose tissue was chosen for the metabolomics analysis because we found this tissue to show the greatest changes in metabolic makers, relative to subcutaneous adipose tissue, as described in Qu et al. (2016). However, the metabolomics profile in the mesenteric adipose tissue of pigs under heat stress was not as clearly separated as in the in vitro differentiated adipocytes. However, increased accumulation of PE(18:0/22:1(13Z)) and PC(16:1(9Z)/20:0) might also help to increase tissue rigidity to counter the fluidization of mesenteric adipose tissue caused by HS. The increased accumulation of 4,8 dimethylnonanoyl carnitine, a fatty acid oxidation intermediate product may reflect impairment of fatty acid oxidation in adipose tissue in HS, hence accumulation of this intermediary product. At present, the potential biological implication of the elevated adenine and L-arginine metabolism is unknown, although increased L-arginine metabolism might indicate increased proteolysis of proteins in adipose tissue of pigs under heat stress. Increased L-arginine might also provide increased level of this precursor for nitric oxide synthesis (Stuehr, 2004), perhaps to increase tissue blood flow (Ardilouze et al., 2004) during HS.

The lower concentration of serum stearic acid and the lower total SFA in heat stressed pigs may suggest an increased withdrawal of saturated fatty acids into tissues, perhaps to be used for increasing tissue rigidity during HS. However, adipose tissue stearic acid and SFA content were not affected by heat stress. Serum fatty acid composition is a balance of fatty acids coming from the diet, adipose tissue lipolysis and tissue uptake. Circulating FFA composition to some extent

reflects the fatty acid composition in adipose tissue (Yew Tan et al., 2015). Thus, lower serum stearic acid and SFA may also reflect reduced lipolysis from adipose tissue. Overall, fatty acid profile adipose tissue was not affected by treatment. This may reflect the in vivo complexity of heat stress adaptation that causes no distinct changes in tissue fatty acid composition. Overall, these results show a clear difference between the in vitro and in vivo settings in the separation of metabolite profile by metabolomics approach.

A limitation of the metabolomics analysis is the lack of a good metabolite database with which the identified metabolites can be compared. The HMDB that we used may not be highly sensitive to omics analyses in pigs. Potential differences in tissue composition between tissues and cells exposed to heat stress versus control may give an incorrect indication that different metabolic pathways were stimulated in heat stress.

In summary, the data provided represent a comprehensive overview of the changes in metabolites and individual fatty acids in adipocytes and mesenteric adipose tissue of pigs under HS. We demonstrate that heat stress induces distinct metabolite profiles. This may have implication for the explanation of the regulation of lipogenesis, fatty acid oxidation and membrane fluidity in adipose tissue during heat stress. However, additional work is needed to further elucidate the role that these metabolites and metabolic pathways play in HS adaptation, and whether they could be targeted for optimization of the adaptive response to HS in pigs.

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Table 6.1 Feed ingredient composition (as-fed basis).

Ingredient	Percentage
Corn	79.75
Soybean meal	1.95
Dried distiller's grains	15.00
Swine white grease	1.00
L-lysine hydrochloride	0.38
L-threonine[1]	0.09
L-tryptophan	0.04
Limstone	1.14
Monocalcium phosphate	0.14
Vitamin premix[2]	0.10
Sodium chloride	0.25
Selenium 270 premix[3]	0.03
Phytase[4]	0.08
Non-sulfur T.M. premix[5]	0.05

[1] 98% L-threonine. [2]Swine vitamin concentration in premix: vitamin A, 544,680 IU/kg; vitamin D_3, 54,448 IU/kg; vitamin E, 3631 IU/kg; menadione (vitamin K), 182 mg/kg; vitamin B_{12}, 3.2 mg/kg; riboflavin, 726 mg/kg; d-pantothenic acid, 1816 mg/kg; niacin, 2723 mg/kg, biotin, 18.1 mg/kg; folic acid, 136 mg/kg; choline, 45,390 mg/kg; pyridoxine, 409 mg/kg; vitamin E, 1816 IU/kg; chromium, 16.3 mg/kg; carnitine, 4805 mg/kg. [3]Selenium 270 premix: 600 mg/kg of selenium as sodium selenite in a mixture of equal portions of ground limestone and ground corn to fill 0.05% of the diet. [4]600 ppm phytase. [5]Non-sulfur trace mineral premix: iron, 51.05%; zinc, 20.73%; manganese, 2.86%; copper, 1.56%; iodine, 0.046%.

Table 6.2 Fatty acid profile of adipocytes in control or elevated temperature.

Fatty acid (%)[1]	Control	Heat stress	SEM	*P*-value
C12:0 (Lauric Acid)	0.23	0.33	0.03	0.415
C14:0 (Myristic Acid)	1.88	2.06	0.04	0.004
C16:0 (Palmitic Acid)	29.82	38.92	0.91	<0.001
C16:1 (Palmitoleic Acid)	17.05	7.28	1.76	<0.001
C18:0 (Stearic Acid)	16.23	18.42	1.42	0.991
C18:1n9c (Oleic Acid)	25.94	20.27	1.11	0.885
C18:2n6c (Linoleic Acid)	3.17	1.92	0.41	0.030
C18:3n6c (γ-linolenic Acid)	0.02	0.01	0.003	0.126
C20:1n9 cis-11-eicosenoic acid (Gadoleic Acid)	1.81	7.48	0.67	<0.001
C22:0 (Behenic acid)	0.27	0.22	0.03	0.121
C20:5n3 (Eicosapentaenoic acid, EPA)	0.40	0.34	0.02	0.010
C24:0 (Lignoceric acid)	0.53	0.38	0.05	0.006
C22:6n3 (Docosahexaenoic acid, DHA)	2.66	2.81	0.06	0.402
Total SFA	48.96	60.33	1.58	<0.001
Total MUFA	44.80	35.03	1.48	<0.001
Total PUFA	6.25	5.08	0.52	0.421
MUFA/SFA	0.92	0.58	0.03	<0.001
PUFA/SFA	0.13	0.08	0.01	0.077

[1]Individual fatty acids are expressed as percentages of the total. (n=5)

Table 6.3 Fatty acid profile of serum of pigs in either control, pair fed or elevated ambient temperature.

	Treatment[2]			Gender			*P*-value		
Fatty acid (%)[1]	Con	PF	HS	Boar	Gilt	SEM	Trt	Gender	Interaction
C12:0 (Lauric Acid)	0.44	0.60	0.87	0.98	0.45	0.10	0.217	0.007	0.933
C14:0 (Myristic Acid)	0.58	0.76	0.49	0.55	0.59	0.04	0.110	0.972	0.655
C16:0 (Palmitic Acid)	25.65	25.77	25.46	25.84	25.40	0.31	0.836	0.771	0.031
C16:1 (Palmitoleic Acid)	0.65	0.72	0.57	0.40	0.80	0.10	0.991	0.030	0.947
C18:0 (Stearic Acid)	29.23^{a}	30.32^{a}	25.07^{b}	28.34	26.92	0.69	0.002	0.236	0.204
C18:1n9t (Elaidic Acid)	0.62	0.48	0.23	0.14	0.61	0.09	0.179	0.014	0.111
C18:1n9c (Oleic Acid)	25.03	26.80	26.55	25.03	26.94	0.40	0.153	0.014	0.682
C18:2n6c (Linoleic Acid)	17.74^{ab}	14.37^{a}	20.68^{b}	18.63	18.20	0.78	0.004	0.522	0.212
C18:3n6c (γ-linolenic Acid)	0.03	0.04	0.04	0.04	0.04	0.006	0.969	0.654	0.080
C20:3n6 (Dihomo-γ-linolenic acid)	0.03	0.14	0.03	0.07	0.04	0.02	0.320	0.156	0.179
Total SFA	57.24^{a}	56.28^{a}	51.4^{b}	54.65	53.66	0.86	0.003	0.131	0.008
Total MUFA	25.55	27.82	27.37	25.80	27.57	0.43	0.288	0.001	0.133
Total PUFA	17.21^{b}	15.91^{b}	21.22^{a}	19.55	18.78	0.77	0.005	0.398	0.001
MUFA/SFA	0.45	0.50	0.54	0.47	0.52	0.01	0.059	0.002	0.079
PUFA/SFA	0.30^{b}	0.29^{b}	0.42^{a}	0.36	0.36	0.02	0.003	0.896	0.001

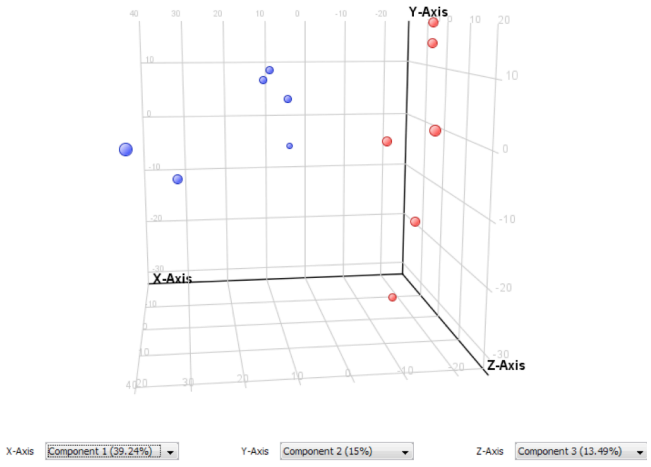
[1]Individual fatty acids are expressed as percentages of the total. [2] Control treatment (CON)； pair-fed treatment (PF); Heat treatment (HS). a;b superscripts indicate significant mean differences between Con, PF and HS treatments, $P < 0.05$. (n=10)

Table 6.4 Fatty acid profile of mesenteric fat of pigs in control, pair fed or elevated ambient temperature.

	Treatment[2]			Gender			*P*-value		
Fatty acid (%)[1]	Con	PF	HS	Boar	Gilt	SEM	Trt	Gender	Interaction
C12:0 (Lauric Acid)	0.09	0.10	0.09	0.10	0.09	0.01	0.922	0.548	0.211
C14:0 (Myristic Acid)	1.70	1.56	1.49	1.53	1.63	0.07	0.421	0.299	0.231
C16:0 (Palmitic Acid)	28.27	28.92	28.24	27.50	29.44	0.64	0.880	0.461	0.176
C16:1 (Palmitoleic Acid)	2.22	1.88	1.68	1.96	1.90	0.13	0.133	0.493	0.231
C18:0 (Stearic Acid)	12.47	12.15	11.88	12.12	12.28	0.63	0.848	0.367	0.667
C18:1n9t (Elaidic Acid)	0.24	0.27	0.24	0.25	0.25	0.02	0.862	0.735	0.654
C18:1n9c (Oleic Acid)	41.78	41.41	41.06	40.77	41.65	0.91	0.171	0.888	0.025
C18:2n6c (Linoleic Acid)	11.78	11.97	13.65	14.11	11.02	0.64	0.318	0.002	0.320
C18:3n3,9,12 α-linolenic (Alpha linolenic Acid)	0.43	0.54	0.59	0.50	0.56	0.02	0.194	0.875	0.671
C20:1n9 cis-11-eicosenoic acid (Gadoleic Acid)	0.79	0.81	0.74	0.71	0.87	0.04	0.720	0.031	0.972
C20:3n6 (Dihomo-γ-linolenic acid)	0.13	0.27	0.23	0.24	0.19	0.04	0.352	0.856	0.107
C20:4n6 (Arachidonic Acid)	0.09	0.10	0.11	0.09	0.10	0.01	0.532	0.630	0.762
Total SFA	42.53	42.73	41.7	41.25	43.44	1.22	0.912	0.414	0.293
Total MUFA	45.03	44.37	43.72	43.69	44.67	1.72	0.884	0.529	0.238
Total PUFA	12.43	12.88	14.58	14.94	11.87	0.92	0.297	0.097	0.543
MUFA/SFA	1.06	1.04	1.05	1.06	1.03	0.06	0.895	0.896	0.226
PUFA/SFA	0.29	0.30	0.35	0.36	0.27	0.02	0.104	0.028	0.846

[1]Individual fatty acids are expressed as percentages of the total. [2] Control treatment (CON)； pair-fed treatment (PF); Heat treatment (HS). (n=10)

Figure 6.1 Principal component analysis (PCA) in in vitro differentiated adipocytes.



PCA analysis, metabolites were separated by temperature using PCA. The axes correspond to x-axis (component 1) (PC1), y-axis(component 2), z-axis (component 3). Data compression and reduced dimensionality was applied when performing the data into components and to form feature vectors in 3 dimensions along the x-, y- and z-axis. (n=5)

Figure 6.2 Abundance of different species of non-polar metabolites showing numbers that are increased or decreased in adipocytes exposed to heat stress (41.5°C) relative to the control (37°C).

A.

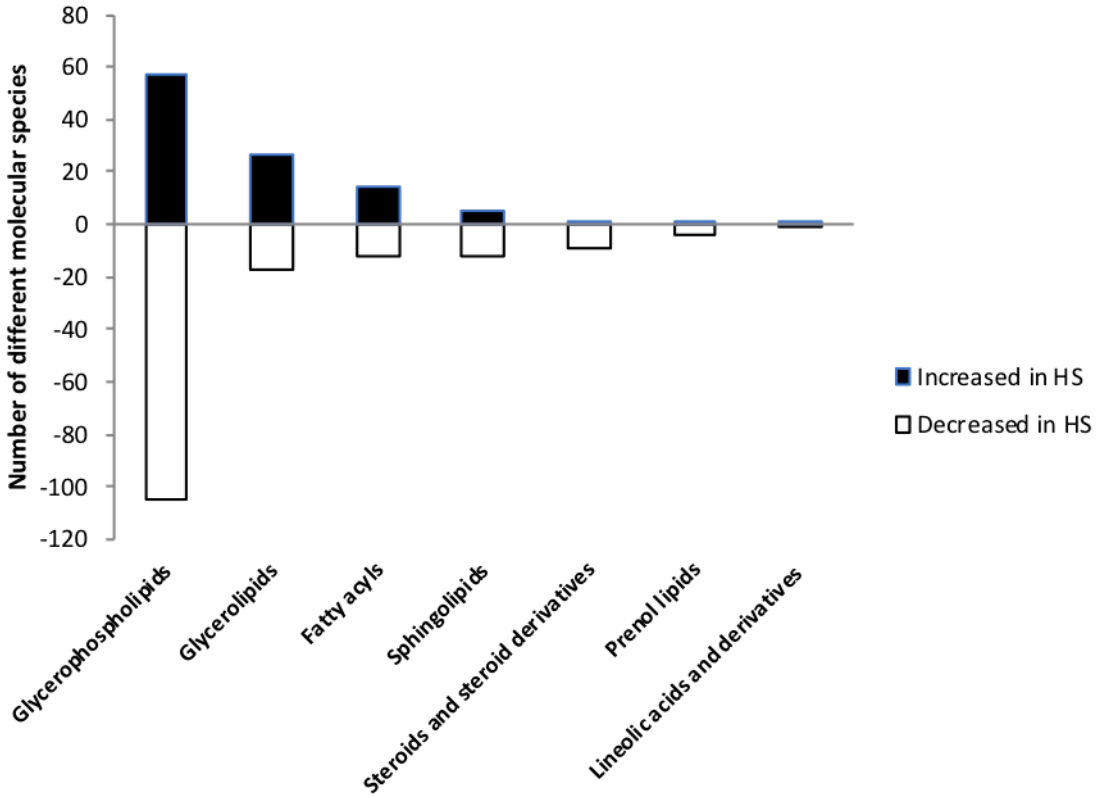


Figure 6.2 Continued

B.

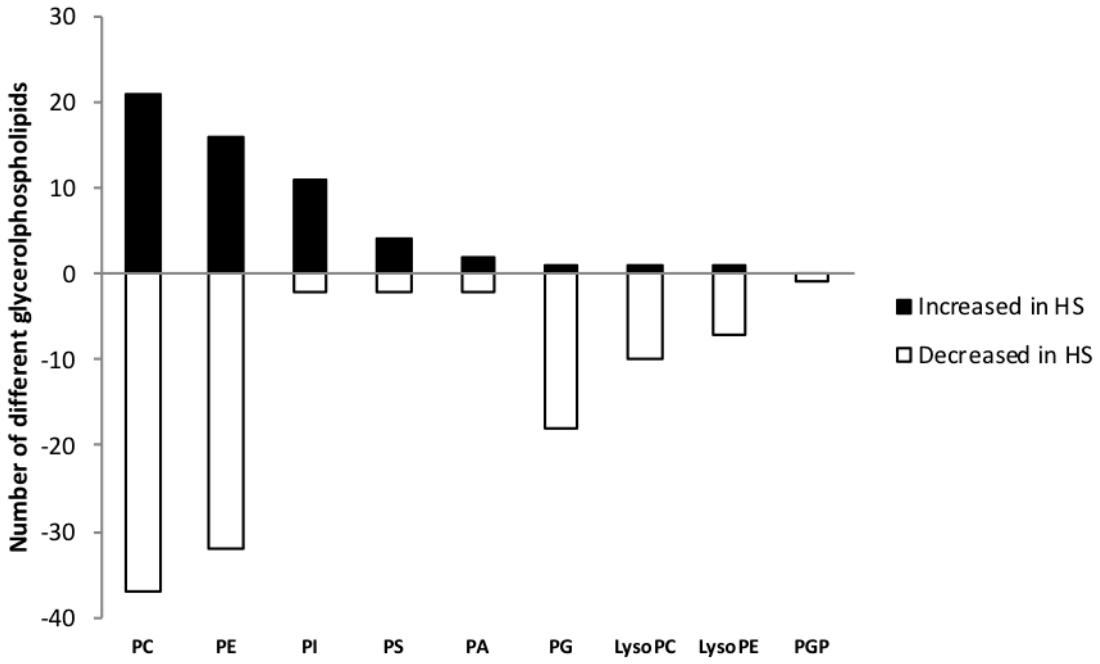
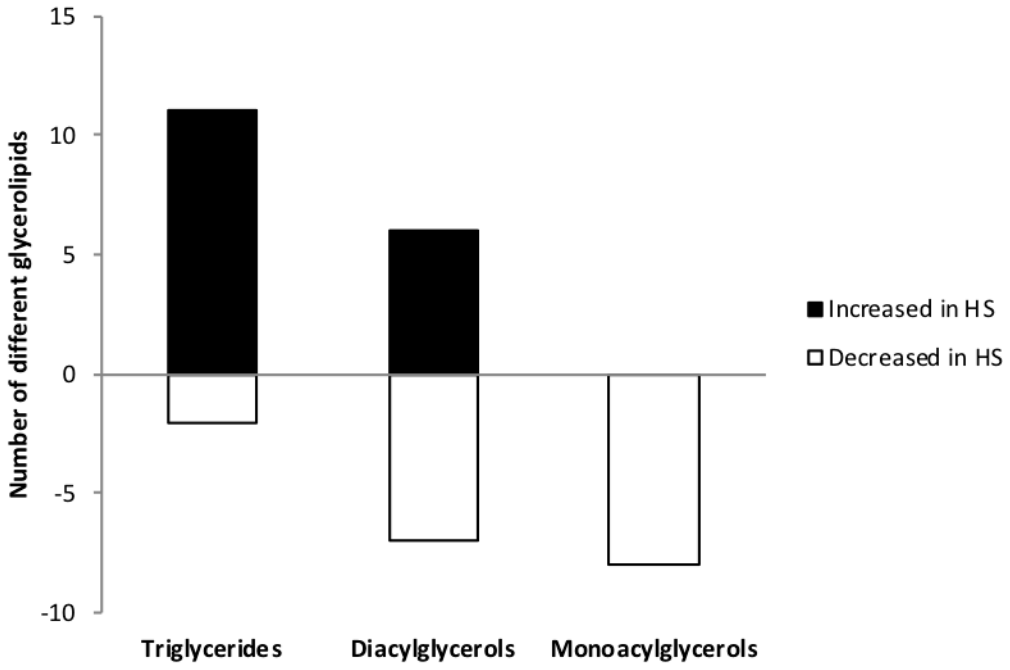


Figure 6.2 Continued

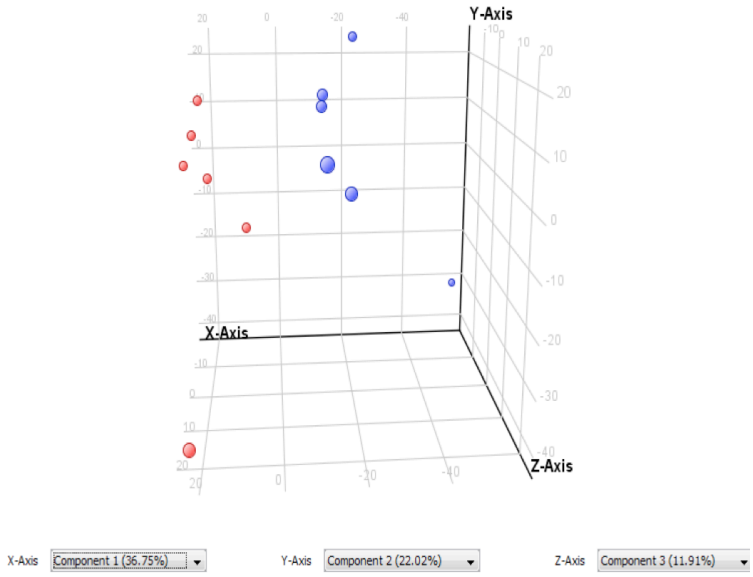
C.



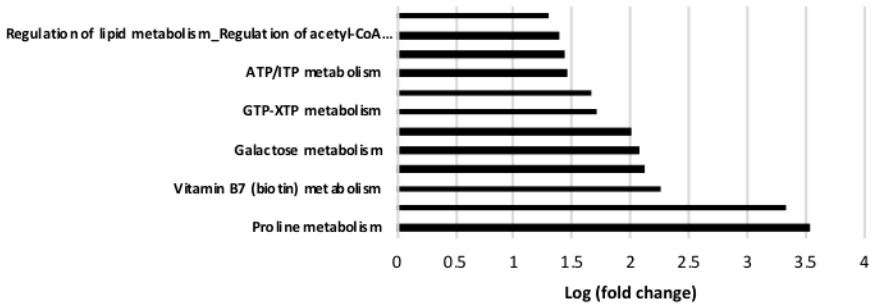
(A) Number of the different molecular species according to their categories. (B) Number of different glycerophospholipids. (C) Number of different glycerolipids. Bars represent number of species (black bars indicate number increased and open bars indicate number decreased in HS compared to control). (n=5)

Figure 6.3 Principal component analysis (PCA), and pathway analysis of metabolomics analysis of polar compounds in in vitro differentiated adipocytes.

A.



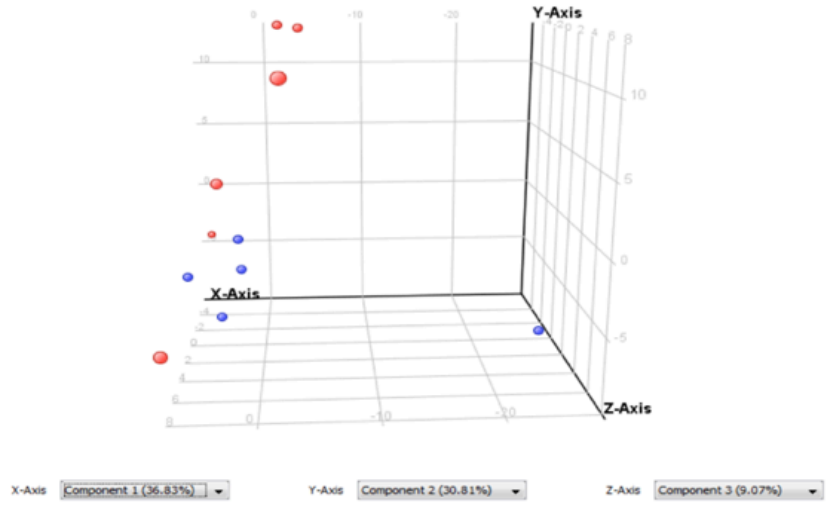
B.



(A) PCA analysis, metabolites were separated by temperature using PCA. The axes correspond to x-axis(component 1) (PC1), y-axis(component 2), z-axis(component 3). Data compression and reduced dimensionality was applied when performing the data into components and to form feature vectors in 3 dimensions along the x-, y- and z-axis. (B) Pathway analysis showing fold upregulation of specific metabolic pathways in HS compared to control. (n=5)

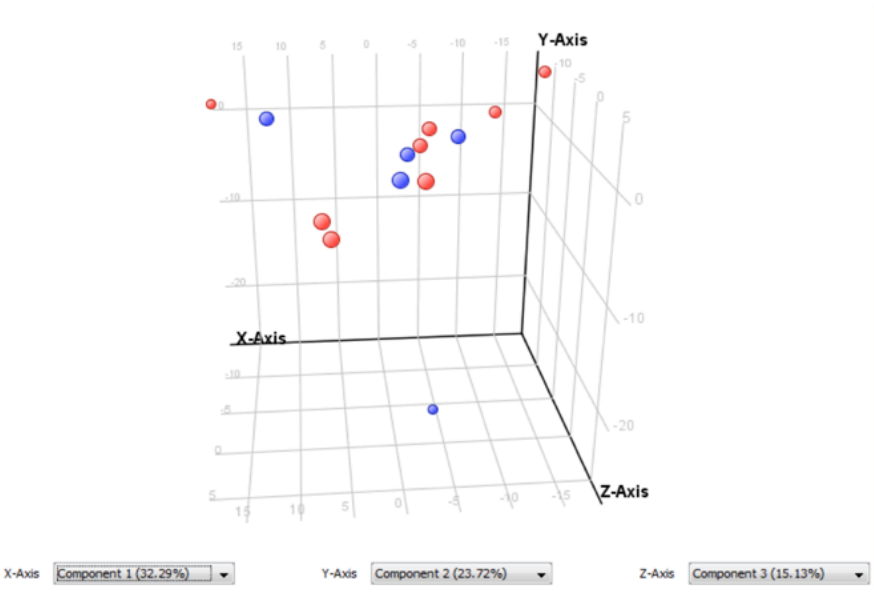
Figure 6.4 Principal component analysis (PCA) and metabolomics analysis of non-polar compounds of pigs kept at normal room temperature (20 °C) and pair-fed (PF) to the feed intake of animals in heat stress (HS) (35 °C) for one week. The axes correspond to x-axis(component 1) (PC1), y-axis(component 2), z-axis(component 3). Data compression and reduced dimensionality was applied when performing the data into components and to form feature vectors in 3 dimensions along the x-, y- and z-axis.

A.



Boars, PF *vs*. HS.

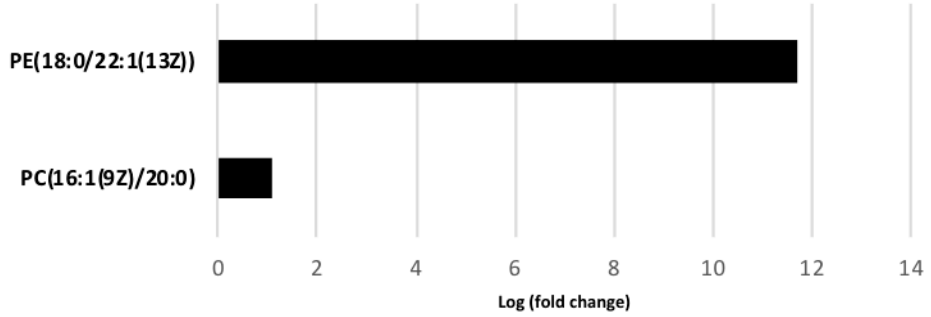
B.



Gilts, PF *vs*. HS.

Figure 6.4 Continued

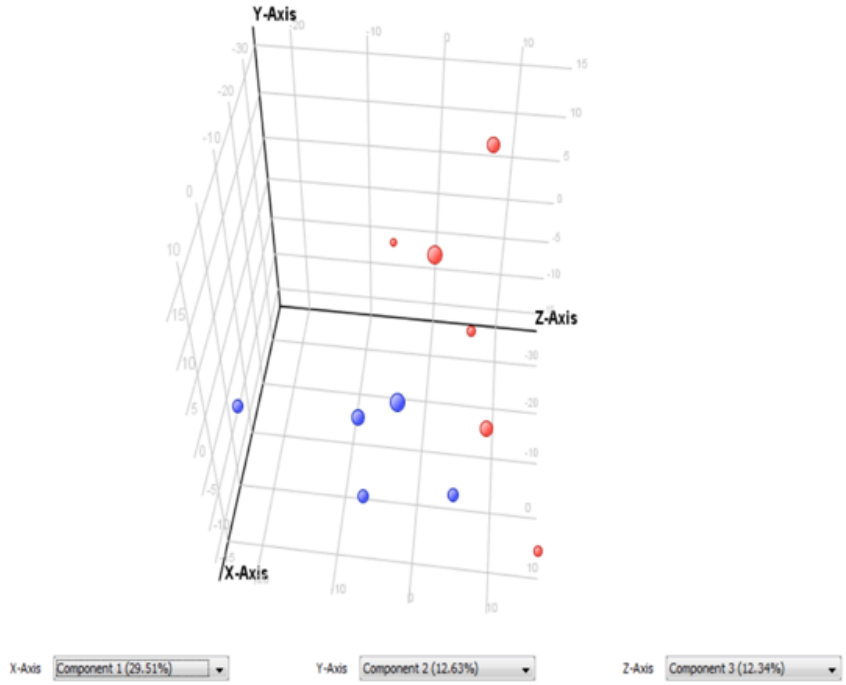
C.



(A). Differences between boars in PF or HS treatments. (B) Differences between gilts in PF or HS treatments (C) Pathway analysis of non-polar metabolites in boars in PF or HS treatments showing pathways that are upregulated in HS relative to PF. (n=5)

Figure 6.5 Principal component analysis (PCA) and metabolomics analysis of polar compounds of pigs kept at normal room temperature (20 °C) and pair-fed (PF) to the feed intake of animals in heat stress (HS) (35 °C) for one week. The axes correspond to x-axis(component 1) (PC1), y-axis(component 2), z-axis(component 3). Data compression and reduced dimensionality was applied when performing the data into components and to form feature vectors in 3 dimensions along the x-, y- and z-axis.

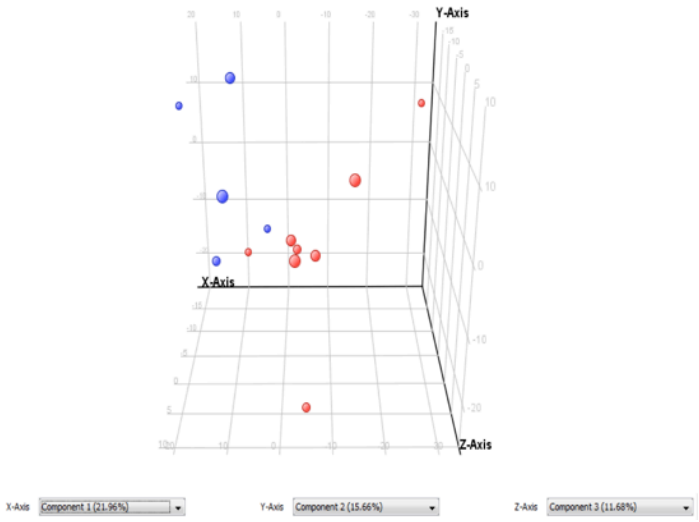
A.



Boars, PF *vs*. HS.

Figure 6.5 Continued

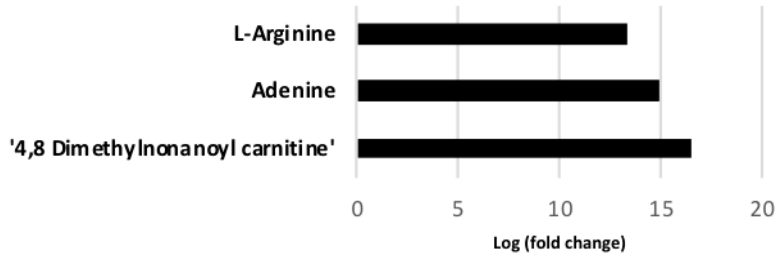
B.



Gilts, PF *vs*. HS.

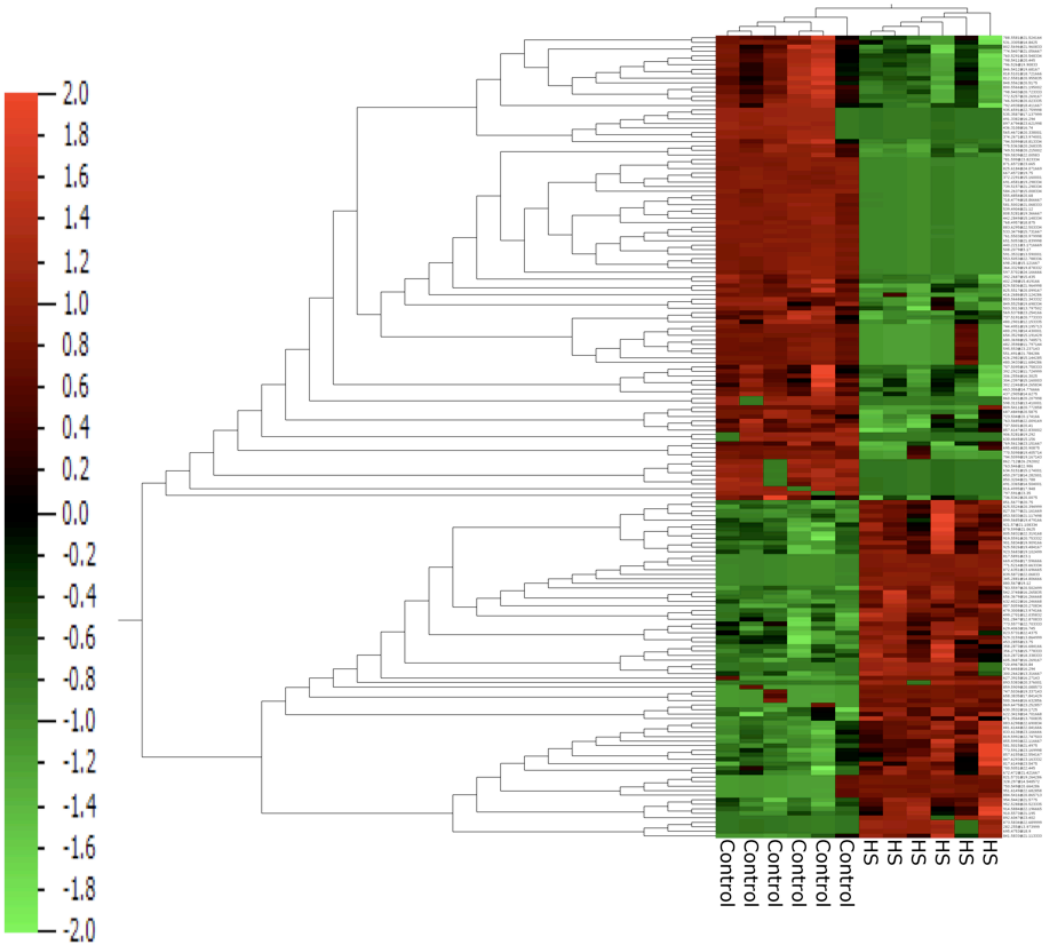
Figure 6.5 Continued

C.



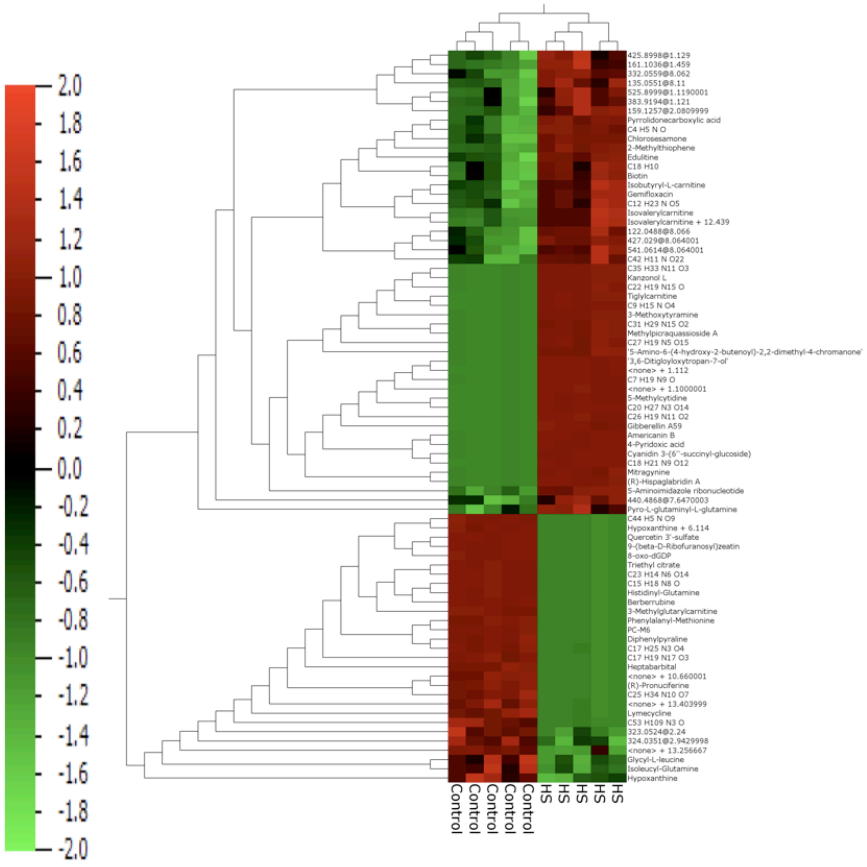
(A). Differences between boars in PF or HS treatments. (B) Differences between gilts in PF or HS treatments (C) Pathway analysis of non-polar metabolites in gilts in PF or HS treatments showing pathways that are upregulated in HS relative to PF. (n=5)

Figure 6.6 Heatmap of pig adipocyte lipidomic.



Heat map shows distinct pattern of abundance between the control (37°C) and heat stress (HS, 41.5°C). Green colors indicate downregulated compounds, red colors indicate upregulated compounds. (n=5)

Figure 6.7 Heatmap of pig adipocyte metabolomics.



Heat map shows distinct pattern of abundance between the control (37°C) and heat stress (HS, 41.5°C). Green colors indicate downregulated compounds, red colors indicate upregulated compounds. (n=5)

CHAPTER 7. SUMMARY

Heat stress results in increased carcass fat content in the pig compared to pair fed animals in thermoneutral temperature. Although precise mechanisms of this effect are unknown, elevated plasma insulin, a lipogenic hormone, during HS may be a contributing factor. Increased insulin concentration may also be responsible for the increased expression and activity of LPL, another adipogenic enzyme, in the adipose tissue of heat stressed pigs. Despite the likely implication of insulin in the increased fat mass in heat stressed pigs, there are significant inconsistencies in the regulation of serum insulin concentrations in pigs under HS suggesting additional mechanisms may be involved. At present, little is known about HS response at the cellular level in the adipocyte. Therefore, the overall objective of experiments described in this thesis was to understand the mechanism of HS response at systemic, tissue and cellular levels. Although our main focus was understanding adipose and adipocyte-specific responses, we also determined responses in both the liver and skeletal muscle. We used both in vitro differentiated pig adipocyte model and in vivo pig model to study effect of exposure to HS.

In Chapter 2, we wanted to study the mechanism of HS effects on porcine adipocytes. Therefore, we used an in vitro adipocyte differentiation model to characterize cellular responses that occur during differentiation of pig adipocytes exposed to HS. Our major finding was that HS directly leads to increased adipogenesis in porcine adipocytes. The mechanisms of this response may include increased mRNA level of transcription factor C/EBPα, and increased mRNA level of fatty acid transport and glyceroneogenic genes (PCK1 and GK1). In Chapter 3, we sought to elucidate the mechanisms of HS response in animals exposed to short-duration (7-d) continuous HS, especially adipose tissue specific responses. We used cross-bred (Ossabaw × Duroc × Landrace) pigs in this study. We found that HS led to increased adipose tissue mRNA and protein level of PCK1, confirming findings in vitro that induction of glyceroneogenesis could be a major metabolic response in adipose tissue during HS. These responses occurred without any alterations in serum glucose, insulin, FFA and triglyceride concentrations, indicating that changes within adipose tissue might not affect systemic responses with respect to regulation of these important metabolites. In Chapter 4, we sought to know if duration of exposure played a

major role in the response to HS. We used cross-bred pigs (Duroc X Yorkshire X Landrace terminal cross) exposed to either acute (24 h) or chronic (7 d) HS. mRNA level changes in adipose tissue, muscle and liver were analyzed. Consistent with findings in Chapters 2 and 3, we also found that HS induced a robust adipose tissue response in favor of increased lipid storage. With the abundance of evidence pointing to an important role of PCK1 in adipose tissue HS response, in Chapter 5 we characterized the role of PCK1 in adipocyte metabolic adaptation to HS, specifically the effect of PCK1 inhibition during heat stress on lipogenesis, mRNA and protein level of ER stress markers and heat shock proteins. Therefore, we conducted in vitro experiments in which the activity of PCK1 was inhibited with 3MPA, and we determined effects on lipid storage and HS and ER stress response during HS. We found that with inhibition of PCK1 during HS, adipocytes were less able to induce adaptive responses such as upregulation of HSP70 and increased triglyceride accumulation, and this exacerbated ER stress during HS. Therefore, PCK1 may function to alleviate ER stress that occurs during HS. In Chapter 6, our goal was to determine the global profile of metabolites and lipid species differentially regulated in HS to see if they could serve as useful biomarkers associated with HS response. This was done with metabolomics analyses and GC analyses for individual fatty acids. Our major finding was that HS induced distinct metabolite profiles. Thus, these metabolites might be involved in the observed changes in lipid metabolism and overall characteristics and function of adipocytes in HS. These changes might be the underlying basis of changes observed in lipogenic potential in the adipocyte and potential changes in fatty acid oxidation and membrane fluidity.

Additional future research on the contribution of some of the identified metabolites will help shed further light on the role of these metabolites in the HS response. More work is also needed on the identity of cellular proteins involved in HS response. A proteomics approach might offer an opportunity for identification of a wide array of proteins that may be involved in the HS response.

In summary, experiments described in this thesis provide evidence that adipose-specific response are an integral part of the whole-body response to HS. The extent to which these responses contribute to whole-body adaptive response to HS are still unknown.

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