Adipose tissue-specific responses reveal an important role of lipogenesis during heat stress adaptation in pigs¹

H. Qu and K. M. Ajuwon²

Department of Animal Sciences, Purdue University, West Lafayette, IN 47907-2054

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 approximately 36% and 64% in HS and AHS treatments respectively, 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 GK mRNA between Con, PF, and HS, although its expression was lower ($P = 0.003$) in AHS vs. HS. Protein abundance of the ER stress marker, CCAT/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 was higher ($P < 0.001$) in the HS than Con and PF treatments. Additionally, expression of PCK1 was lower ($P = 0.039$) in AHS vs. HS. Expression 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 stress adaptation.

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

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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

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²Corresponding author: kajuwon@purdue.edu

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markers, especially phosphoenolpyruvate carbox-kinase (**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 gene expression 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 heat stress adaptation in pigs.

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 (**PACUC**). A total of 40 crossbred barrows, with mean initial body weight of 72.68 ± 2 kg, were randomly allocated (10/treatment) to four treatments: control (**Con**) treatment with ad libitum feeding, with room temperature set at $20^\circ\text{C} \pm 1^\circ\text{C}$ and 53%–78% relative humidity (RH) for 7 d; **HS** treatment with room temperature set at $35^\circ\text{C} \pm 1^\circ\text{C}$, RH at 45%–65% with ad libitum feed intake (**FI**) for 7 d (chronic exposure); pair-fed (**PF**) group with same room temperature and RH as the Con treatment, and pair-fed to the FI of heat **HS** group for 7 d; acute heat

stress (**AHS**) treatment with room temperature at $35^\circ\text{C} \pm 1^\circ\text{C}$ and RH at 45%–65% with ad libitum FI 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. FI and skin temperature were measured daily during the experiment. Skin temperatures were measured using an infrared non-contact digital thermometer (model WIC-276664; Walgreens, Deerfield, IL). RTs were measured with a digital rectal thermometer (model KD-113; Walgreens). Respiration rate (**RR**) was measured three times at the end of the experiment (day 1 for

Table 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	0.38
L-threonine ¹	0.09
L-tryptophan	0.04
Limestone	1.14
Monocal 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
Calculated composition:	
CP, %	12.0
ME, Mcal/kg	3.37

¹98% L-threonine.

²Swine vitamin premix: vitamin A, 544,680 IU/kg; vitamin D3, 54,448 IU/kg; vitamin E, 3631 IU/kg; menadione (vitamin K), 182 mg/kg; vitamin B12, 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.

³Selenium 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.

⁴600 ppm phytase.

⁵Non-sulfur trace mineral premix: iron, 51.05%; zinc, 20.73%; manganese, 2.86%; copper, 1.56%; iodine, 0.046%.

AHS and day 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 day 0, day 1 (AHS pigs only), and day 7 of the experiment through jugular venipuncture. Plasma was separated from whole blood through centrifugation at $10,000 \times g$ for 10 min at 4 °C. Pigs were euthanized after sedation with intramuscular injection of atropine, tiletamine-zolazepam and xylazine, and CO₂ asphyxiation, followed by pneumothorax and cardiectomy while anesthetized. After sacrifice, adipose tissues, subcutaneous (collected from the middle layer at the 10th rib) and mesenteric (from the ileum), longissimus dorsi (LD) and semitendinosus (STD) muscles and liver were collected on day 1 (AHS) or day 7 (Con, PF, and HS treatments) of experiment. Tissues were snap frozen in liquid nitrogen immediately after collection and stored in -80 °C before RNA and protein extraction.

Gene Expression Analysis

Expression of metabolic and inflammatory genes in subcutaneous adipose tissue, liver, and muscle was determined by real-time polymerase chain reaction (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) 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 $\Delta\Delta C_t$ method.

Western Blot Analysis

Tissues were homogenized in 1× radio-immunoprecipitation assay 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], 0.1% Triton X [Sigma-Aldrich]) supplemented with commercial protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Homogenates were centrifuged at $10,000 \times g$ 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

Table 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'

¹aP2 = 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.

(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 house-keeper (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). Plasma free fatty acid (FFA) was determined using a Non-esterified fatty acid kit (NEFA) kit (Wako Diagnostics, Richmond, VA). Blood urea nitrogen (BUN) was measured with BUN colorimetric detection kit (Arbor Assays, Ann Arbor, MI). Plasma glucose was measured with a commercially available kit (Wako Diagnostics). Plasma insulin was determined using the Mercodia porcine insulin ELISA kit (Mercodia, Uppsala, Sweden).

Statistical Analyses

Data were analyzed to determine differences between Con vs. PF vs. HS, and between HS and AHS by one-way analysis of variance with treatment and animal considered fixed and random effects, respectively. This was followed by Tukey's mean separation test (SAS Inst. Inc., Cary, NC). 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 $\pm$ standard error of the mean.

RESULTS

Animal Performance

FI of the PF, HS, and the AHS treatments were lower ($P < 0.05$) than Con in the first 24 h of the study (Fig. 1A). After 7 d, FI was decreased by

approximately 36% in HS treatment compared with Con ($P < 0.05$; Fig. 1B). BW change (Δ BW) was positive and similar in PF and HS pigs, but less than in the Con pigs ($P < 0.01$; Table 3). The HS treatment gained a total of 1.8 ± 0.2 kg, whereas the AHS treatment resulted in weight loss of approximately -1.4 ± 0.1 kg. Skin temperature was higher ($P < 0.001$) by approximately 2°C in HS and AHS compared with Con and PF in the first 24 h of the study (Fig. 1C). This difference remained between HS, Con, and PF after 7 d of the study (Fig. 1D). There was greater and positive RT change in HS vs. Con. ($P < 0.043$) (Table 3). However, there was no difference in this change between HS and AHS ($P = 0.731$). RR was 2-fold higher ($P = 0.023$) in HS compared to Con and PF (Fig. 1E). The AHS treatment had significantly lower ($P < 0.037$) RR compared with HS.

Plasma Metabolites

Plasma TAG was not different between Con, PF, and HS and between HS and AHS (Fig. 2A). Although plasma FFA was not different between Con, PF, and HS, it was elevated 2-fold in AHS compared to HS ($P = 0.031$; Fig. 2B). However, there was no treatment effect on plasma glucose (Fig. 2C). Insulin concentration was lower in PF than Con ($P = 0.036$, Fig. 2D), but there was no difference between HS and AHS ($P = 0.698$). There was no treatment effect on glucose to insulin ratio (Fig. 2E). In addition, BUN concentration was increased ($P = 0.008$) in HS compared to Con and PF (Fig. 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 expression in HS compared with Con and PF ($P = 0.003$, Table 4). Expression 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. 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, and $P = 0.048$, Fig. 3B), and also higher in HS than AHS. However, there was no difference in GK mRNA between Con, PF, and HS (Table 4), although its expression was lower ($P = 0.003$) in AHS vs. HS. The mRNA expression of aP2 was elevated in HS compared to PF (1.5-fold, $P = 0.035$,

Figure 1. Heat stress (HS) effects on average daily FI after 24 h (A), and 7 d (B) on treatments; skin temperature after 24 h (C) and 7 d (D) on treatments; respiration rate (E) on day of sacrifice in control (Con, 20 °C with ad libitum FI for 7 d), pair-fed (PF, 20 °C with pair feeding to heat stress treatment for 7 d), heat stress (35°C with ad libitum FI for 7 d) environments and acute heat stress (AHS) (35 °C with ad libitum FI for 24 h). ^{a,b}Different superscript letters indicate significant mean differences ($P < 0.05$). Bars represent means $\pm$ standard error of the mean.

Table 3. Effects of heat stress on performance in pigs

Variable ¹	Control	PF	HS	AHS	SEM	<i>P</i> -value C vs. PF vs. HS	<i>P</i> -value HS vs. AHS
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
Δ BW, 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
Δ RT, °C	-0.52 ^b	-0.19 ^{a,b}	0.22 ^a	0.27	0.13	0.043	0.731

¹FBW = final body weight; FRT = final rectal temperature; IBW = initial body weight; Δ BW = body weight change; IRT = initial rectal temperature; Δ RT = rectal temperature change; Different subscripts represent differences between Control (C), pair-fed (PF), and 7 d heat stress (HS) at $P < 0.05$. AHS = acute (24 h) heat stress.

Table 4). There was no difference in the expression of GLUT4 between Con, PF and HS ($P = 0.046$, **Table 4**), although its expression was lower in AHS vs. HS ($P < 0.011$). Inflammatory cytokines were differentially regulated. Gene expression of IL-6 was higher in HS than Con and PF ($P < 0.001$, **Table 4**), and its expression was also lower in AHS

relative to HS ($P = 0.51$). However, the mRNA expression of TNF- α was lower in HS and PF than Con ($P < 0.001$, **Table 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. 3C**).

Figure 2. Heat stress (HS) effects on plasma metabolites in control (Con, 20 °C with ad libitum FI for 7 d), pair-fed (PF, 20 °C with pair feeding to heat stress treatment for 7 d), heat stress (35 °C with ad libitum FI for 7 d) environments and acute heat stress (AHS) (35 °C with ad libitum FI for 24 h). 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 $\pm$ standard error of the mean. ^{a,b}Different superscript letters indicate significant mean differences between Con, PF, and HS ($P < 0.05$).

Mesenteric Fat-specific Responses to HS

There was no difference in HSP70 mRNA in treatments ($P = 0.361$, Table 5). Similarly, there was no treatment effect on the HSP70 protein level (Fig. 4A). Similar to findings in the subcutaneous adipose tissue, gene expression of PCK1 was higher in the HS than Con and PF treatments ($P < 0.001$, Table 5). Additionally, expression of PCK1 was lower ($P = 0.039$) in AHS vs. HS. However, there was no significant treatment difference in PCK1 protein expression (Fig. 4B). Expression of LPL was lower ($P = 0.036$) in AHS compared with HS

(Table 5). Leptin expression was lower ($P = 0.012$) in PF than Con (Table 5). There was no treatment effect on adiponectin expression. Furthermore, protein expression of CHOP was not significantly affected by treatment (Fig. 4C).

Muscle and Liver-specific Responses to HS

Gene expression 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 6). However,

Table 4. Effects of heat stress on subcutaneous fat gene expressions in pigs

Gene ¹	Control	PF	HS	AHS	SEM	<i>P</i> -value	<i>P</i> -value
						C vs. PF vs. HS	HS vs. AHS
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 ^{a,b}	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 ^b	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

¹HSP 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 (C), pair-fed (PF), and 7 d heat stress (HS) at $P < 0.05$. AHS = acute (24 h) heat stress.

HSP70 expression was lower ($P < 0.003$) in the LD of pigs in AHS compared to those in HS. There was a higher LPL gene expression in STD muscle in HS than other treatments ($P < 0.001$, Table 6). Expression 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 expression in LD muscle (Table 6). Expression of FAS was lower in AHS compared to HS ($P = 0.034$). Pigs in the PF treatment had a higher liver PCK1 gene expression than other treatments ($P = 0.047$, Table 7). Protein levels of HSP70, PCK1, and CHOP were not significantly affected by treatment in the LD muscle and liver (Fig. 5 and 6).

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 in heat stress will ingest copious amounts of water, increase recreational water use and reduce FI and activity (Kellner et al., 2016; Lucy and Safranski, 2017). At the cellular level, heat stress results in increased expression 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 expression of critical metabolic genes during different durations of heat stress exposure are still incompletely understood. Higher skin and RTs, and a higher RR, 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 FI and reduced BW 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 level 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 7 d

Figure 3. Heat stress (HS) effects on protein expression in subcutaneous fat in control (Con, 20 °C with ad libitum FI for 7 d), pair-fed (PF, 20 °C with pair feeding to heat stress treatment for 7 d), heat stress (35 °C with ad libitum FI for 7 d) and acute heat stress (AHS) (35 °C with ad libitum FI for 24 h). (A) Heat shock protein 70 (HSP70). (B) Phosphoenolpyruvate carboxykinase (PCK1). (C) CCAAT-enhancer-binding protein homologous protein (CHOP). Bars represent means $\pm$ standard error of the mean. ^{a,b}Different superscript letters indicate significant mean differences between Con, PF, and HS ($P < 0.05$).

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 expression 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 day 7. The elevation of PCK1 gene expression 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

Table 5. Effects of heat stress on mesenteric fat gene expression in pigs

Gene ¹	Control	PF	HS	AHS	SEM	<i>P</i> -value	<i>P</i> -value
						C vs. PF vs. HS	HS vs. AHS
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 ^{a,b}	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

¹HSP 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 (C), pair-fed (PF), and 7 d heat stress (HS) at $P < 0.05$. AHS = acute (24 h) heat stress.

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 level, 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 FI. 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 FI reduction. For example, in contrast to the induction of expression of PCK1 in adipose tissue in the HS group, pair-feeding led to induction of PCK1 gene expression in the liver of PF pigs. The elevation of PCK1 expression 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 expression 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 gene expression in the liver (Barthel and Schmoll, 2003). Therefore, the lowering of serum concentration of insulin would remove the negative effect of insulin

on PCK1 expression 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 expression 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 expression 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 includes 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 expression 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 prevent protein denaturation (Verghese et al., 2012). The cytoprotective role of HSP70 also depends on its ability to inhibit apoptosis (Samali and Orrenius, 1998;

Figure 4. Heat stress (HS) effects on protein expression in mesenteric fat in control (Con, 20 °C with ad libitum FI for 7 d), pair-fed (PF, 20 °C with pair feeding to heat stress treatment for 7 d), heat stress (35 °C with ad libitum FI for 7 d), and acute heat stress (AHS) (35 °C with ad libitum FI for 24 h). (A) Heat shock protein 70 (HSP70). (B) Phosphoenolpyruvate carboxykinase (PCK1). (C) CCAAT-enhancer-binding protein homologous protein (CHOP). Bars represent means $\pm$ standard error of the mean. ^{a,b}Different superscript letters indicate significant mean differences between Con, PF, and HS ($P < 0.05$).

Garrido et al., 2003). Therefore, these results may suggest that regulation of HSP70 mRNA expression is tissue specific. Furthermore, HSP70 protein expression 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 1 day 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 is 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 expression was not induced in AHS. CHOP is an endoplasmic reticulum (ER) stress marker. Heat stress results

Table 6. Effects of heat stress on expression of genes in the STD² and LD³ muscle of pigs

	Gene ¹	Control	PF	HS	AHS	SEM	<i>P</i> -value C vs. PF vs. HS	<i>P</i> -value HS vs. AHS
STD	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
LD	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

¹HSP 70 = heat shock protein 70; PCK1 = phosphoenolpyruvate carboxykinase 1; GK = glycerol kinase; FAS = fatty acid synthase; LPL = lipoprotein lipase

²STD = semitendinosus muscle; ³LD = longissimus dorsi muscle. Different subscripts represent differences between Control (C), pair-fed (PF), and 7 d heat stress (HS) at $P < 0.05$. AHS = acute (24 h) heat stress.

Table 7. Effects of heat stress on expression of genes in the liver of pigs

Variable ¹	Control	PF	HS	AHS	SEM	<i>P</i> -value C vs. PF vs. HS	<i>P</i> -value HS vs. AHS
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

¹HSP 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 (C), pair-fed (PF), and 7 d heat stress (HS) at $P < 0.05$. AHS = acute (24 h) heat stress.

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 expression 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 expression 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 expression during heat stress between the two genotypes.

Heat stress increases IL-6 gene expression 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 expression 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 expression was

Figure 5. Heat stress (HS) effects on protein expression in liver in control (Con, 20 °C with ad libitum FI for 7 d), pair-fed (PF, 20 °C with pair feeding to heat stress treatment for 7 d), heat stress (35 °C with ad libitum FI for 7 d) and acute heat stress (AHS) (35 °C with ad libitum FI for 24 h). (A) Heat shock protein 70 (HSP70). (B) Phosphoenolpyruvate carboxykinase (PCK1). (C) CCAAT-enhancer-binding protein homologous protein (CHOP). Bars represent means $\pm$ standard error of the mean. ^{a,b}Different superscript letters indicate significant mean differences between Con, PF, and HS ($P < 0.05$).

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 expression 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 TNF α in adipose tissue heat stress may limit its lipolytic effect, further helping

Figure 6. Heat stress (HS) effects on protein expression in longissimus dorsi muscle in control (Con, 20 °C with ad libitum FI for 7 d), pair-fed (PF, 20 °C with pair feeding to heat stress treatment for 7 d), heat stress (35 °C with ad libitum FI for 7 d), and acute heat stress (AHS) (35 °C with ad libitum FI for 24 h). (A) Heat shock protein 70 (HSP70). (B) Phosphoenolpyruvate carboxykinase (PCK1). (C) CCAAT-enhancer-binding protein homologous protein (CHOP). Bars represent means $\pm$ standard error of the mean. ^{a,b} Different superscript letters indicate significant mean differences between Con, PF and HS ($P < 0.05$).

to preserve adipose tissue. This is similar to the reduction in expression 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 FI 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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