Am J Physiol Regul Integr Comp Physiol 315: R1096–R1106, 2018.
First published September 26, 2018; doi:10.1152/ajpregu.00404.2017.

RESEARCH ARTICLE | *Exploiting Environmental Factors to Improve Health and Performance*

Heat stress decreases metabolic flexibility in skeletal muscle of growing pigs

Lidan Zhao,[1] Ryan P. McMillan,[2] Guohao Xie,[1] Samantha G. L. W. Giridhar,[1] Lance H. Baumgard,[3] Samer El-Kadi,[1] Joshua Selsby,[3] Jason Ross,[3] Nicholas Gabler,[3] Matthew W. Hulver,[2,4] and Robert P. Rhoads[1]

[1]*Department of Animal and Poultry Sciences, Virginia Tech University, Blacksburg, Virginia;* [2]*Virginia Tech Metabolic Phenotyping Core, Virginia Tech University, Blacksburg, Virginia;* [3]*Department of Animal Science, Iowa State University, Ames, Iowa; and* [4]*Department of Human Nutrition, Foods and Exercise, Virginia Tech University, Blacksburg, Virginia*

Submitted 9 November 2017; accepted in final form 18 September 2018

Zhao L, McMillan RP, Xie G, Giridhar SG, Baumgard LH, El-Kadi S, Selsby J, Ross J, Gabler N, Hulver MW, Rhoads RP. Heat stress decreases metabolic flexibility in skeletal muscle of growing pigs. *Am J Physiol Regul Integr Comp Physiol* 315: R1096–R1106, 2018. First published September 26, 2018; doi:10.1152/ajpregu.00404.2017.—Heat-stressed pigs experience metabolic alterations, including altered insulin profiles, reduced lipid mobilization, and compromised intestinal integrity. This is bioenergetically distinct from thermal neutral pigs on a similar nutritional plane. To delineate differences in substrate preferences between direct and indirect (via reduced feed intake) heat stress effects, skeletal muscle fuel metabolism was assessed. Pigs (35.3 ± 0.8 kg) were randomly assigned to three treatments: thermal neutral fed ad libitum (TN; 21°C, $n = 8$), heat stress fed ad libitum (HS; 35°C, $n = 8$), and TN, pair-fed/HS intake (PF; $n = 8$) for 7 days. Body temperature (T_B) and feed intake (FI) were recorded daily. *Longissimus dorsi* muscle was biopsied for metabolic assays on *days −2*, *3*, and *7* relative to initiation of environmental treatments. Heat stress increased T_B and decreased FI ($P < 0.05$). Heat stress inhibited incomplete fatty acid oxidation and glucose oxidation ($P < 0.05$). Metabolic flexibility decreased in HS pigs compared with TN and PF controls ($P < 0.05$). Both phosphofructokinase and pyruvate dehydrogenase (PDH) activities increased in PF ($P < 0.05$); however, TN and HS did not differ. Heat stress inhibited citrate synthase and β-hydroxyacyl-CoA dehydrogenase (β-HAD) activities ($P < 0.05$). Heat stress did not alter PDH phosphorylation or carnitine palmitoyltransferase 1 abundance but reduced acetyl-CoA carboxylase 1 (ACC1) protein abundance ($P < 0.05$). In conclusion, HS decreased skeletal muscle fatty acid oxidation and metabolic flexibility, likely involving β-HAD and ACC regulation.

heat stress; metabolism; muscle; pig

INTRODUCTION

Heat-related disorders occur due to elevated environmental temperature exposure (classical heat stress) or as a result of exercise (exertional heat stress) (51). Dysregulated body temperature disturbs biological systems, causing effects ranging from reduced performance to heat illnesses (6). Despite extensive research in the area of heat stress, relatively little is known on the metabolic and biochemical changes that occur during heat exposure. In particular, it is unclear why certain populations are less tolerant to heat than others. Diabetics for example, have a higher risk of suffering from heat-related illness than nondiabetics, and death rates among diabetics increases significantly during the summer months (10, 45). A metabolic characteristic of animals under a reduced plane of nutrition is decreased blood insulin and/or systemic insulin sensitivity (4). However, heat-stressed animals, despite voluntarily reducing feed intake, paradoxically have increased basal insulin as well as stimulated insulin responses (5, 50). Although such whole body metabolic adaptions to a heat load have been studied recently, investigating the effects of heat stress on fuel utilization, particularly in skeletal muscle, the largest and one of the most metabolically active tissues, is still limited.

Heat stress alters performance by a variety of mechanisms. For reasons not yet clarified or explained, circulating nonesterified fatty acid (NEFA) concentrations are reduced during heat stress, even in the presence of elevated stress hormones (epinephrine, glucagon, and cortisol) that are well-known potent lipolytic agents (12, 50). Heat stress, especially that caused by exercise in the heat, is suggested to cause a shift toward carbohydrate utilization at the expense of fatty acids, albeit not necessarily due to increased skeletal muscle glucose uptake (13, 15, 21, 23). Muscle lactate accumulation has been reported in humans and dogs during exercise in the heat, indicating an increase in the conversion of pyruvate to lactate perhaps in lieu of complete glucose oxidation (14, 23, 32, 52). The pyruvate dehydrogenase (PDH) complex plays a central role in alternatively controlling the fate of carbohydrate versus fatty acid oxidation by regulating entry of glycolytic products into the tricarboxylic acid cycle in mammalian cells (28), but the effect of heat on the regulation of this complex and substrate flexibility remains unclear.

Energy metabolism and substrate utilization are highly coordinated interorgan processes with preferred fuel(s) differing between tissues. Because of the large contribution of skeletal muscle to body mass, substrate metabolism within skeletal muscle represents a significant component of whole body energy homeostasis (26). To delineate between the direct and indirect (via changes in feed intake) effects of heat stress on skeletal muscle metabolism, fuel substrate metabolism was assessed during heat stress or reduced feed intake conditions. We hypothesized that heat stress may change the muscle

Address for reprint requests and other correspondence: R. P. Rhoads, Jr., Dept. of Animal and Poultry Sciences, Virginia Tech University, Blacksburg, VA 24061 (e-mail: rhoadsr@vt.edu).

capacity to use glucose or lipids and the flexibility to switch between their use.

MATERIALS AND METHODS

Animals and experimental design. All pigs were handled in accordance with procedures approved by the Virginia Tech Animal Care and Use Committee. Twenty-four cross-bred (landrace × large white) barrows with an average initial body weight of 35.3 ± 0.8 kg were allowed to acclimate to the experimental housing for 3 days. Pigs were randomly assigned to three groups: thermal neutral fed ad libitum (TN; 21°C), heat stress fed ad libitum (HS; 35°C), and pair-fed/HS intake (PF; 21°C) for 7 days. The mean initial body weights of TN, HS, and PF were 34.5, 36.0, and 35.2 kg, respectively. Pigs were housed individually. All pigs were fed a soybean- and corn-based meal formulated to meet or exceed National Research Council requirements. Pigs were fed twice a day at 8 AM and 6 PM. Pigs had free access to water throughout the experiment. Feed intake was recorded daily starting from 3 days before the thermal challenge. The average feed intake of 3 days before thermal challenge was calculated as a baseline. The percentage of feed intake decrease in the HS pigs was calculated by (daily feed intake after thermal challenge − base line) × 100/base line − 1. The amount of feed given to PF controls was calculated based on the percentage decrease in the HS pigs. Body weight was recorded on *days −2* (2 days before thermal challenge), *3*, and *7*. Longissimus dorsi muscle was biopsied on *days −2*, *3*, and *7* after isoflurane anesthesia. Pigs were fasted at midnight before the biopsy. Approximately 75 mg of muscle were used immediately for metabolic measurements. The rest of muscle was snap-frozen in liquid nitrogen and stored at −80°C until analysis.

Surgery and plasma parameters. After 3 days of acclimation, under general anesthesia using sterile techniques, pigs were surgically fitted with indwelling catheters in the carotid artery and jugular vein, as described previously (9). Pigs were also fitted with an iButton sensor in the intraperitoneal cavity for body temperature measurement. Briefly, the iButton sensor was tied with an ~30-cm nonabsorbable polyamide suture, affixed with epoxy glue, and allowed to completely dry for 24 h. A 5-cm incision ~6 cm to the left of the linea alba on the abdomen skin of the pig was made. The abdominal muscles were cut with a blunt scissor until the peritoneum was punctured. The iButton sensor was sterilized with ethanol and rinsed with sterile water and then placed into the intraperitoneal cavity. The iButton sensor was attached to the peritoneum and abdominal muscle wall with suture, and then the peritoneal puncture, muscle layer, and the incision were closed with suture. Temperature loggers (ibutton thermochrons; Maxim, Dallas, TX) were used to record body temperature every hour. The average of 24-h temperature was calculated as daily body temperature.

Pigs were allowed to recover for 3 days postsurgery before the initiation of treatments. On biopsy days, blood samples were collected in lithium heparin tubes via catheters immediately after the biopsy. The blood samples were centrifuged at 1,500 *g* at 4°C for 25 min. Blood plasma was collected after centrifugation and stored at −80°C for future analyses. Insulin concentrations were measured using a porcine insulin ELISA kit (the intra-assay CV was 3.5%, and the interassay CV was 4.3%; Mercodia). Other blood parameters were measured at the Virginia-Maryland Regional College of Veterinary Medicine using a Beckman-Coulter AU-480 with ISE Chemistry System.

Skeletal muscle biopsy. Pigs were fasted for 8 h before the biopsy and placed under general anesthesia using isoflurane. The location of the first biopsy was taken approximately at the first lumbar vertebra. The location was determined by following the curvature of the last rib of the animal to where it meets the vertebral column and then moved ~2 cm toward the posterior of the animal and ~2 cm lateral from midline of the animal to collect the biopsy. For subsequent biopsies, the location moved in a straight-line ~3 cm toward the posterior of the animal from the previous biopsy site. Skeletal muscle biopsy sites were shaved and aseptically cleaned with betadine and 70% isopropanol. An incision of ~5 cm in length was made in the skin. A 10-gauge × 9 cm long Vacora Bard Biopsy Instrument was inserted through the incision at a 45° angle to the orientation of the Longissimus lumborum muscle fibers to collect the biopsy. After the tissue sample collection, incision sites were sutured (Ethicon; MWI Veterinary Supply, Glendale, AZ), cleaned with 70% isopropanol, and disinfected with an aerosol bandage (Allushield; Valley Vet, Maryville, KS). The first biopsy (~75 mg) of muscle was used immediately for metabolic measurements of substrate oxidation and metabolic flexibility. The remainder of the muscle biopsy was snap-frozen in liquid nitrogen and stored at −80°C until analysis.

Substrate oxidation and metabolic flexibility. Palmitate ([1-^{14}C]palmitic acid), glucose ([U-^{14}C]glucose), and pyruvate ([1-^{14}C]pyruvate) oxidation were performed as previously described (17, 47). Skeletal muscle samples used to assess substrate metabolism and metabolic flexibility were immediately placed in SET buffer (0.25 M sucrose, 1 mM EDTA, 0.01 M Tris·HCl, and 2 mM ATP) and stored on ice until homogenization (~25 min). Each skeletal muscle sample was minced with scissors, transferred to a glass homogenization tube, and homogenized on ice using a Teflon pestle (12 passes at 150 rpm). The sample rested on ice for ~30 s, and the homogenization steps were repeated. The homogenate was transferred to an Eppendorf tube, and a fresh sample was used to measure substrate oxidation and metabolic flexibility. Palmitate oxidation was assessed by measuring and summing $^{14}CO_2$ production and ^{14}C-labeled acid-soluble metabolites from the oxidation of [1-^{14}C]palmitic acid. Glucose oxidation was assessed by measuring $^{14}CO_2$ production from the oxidation of [U-^{14}C]glucose, and pyruvate oxidation was assessed by measuring $^{14}CO_2$ production from the oxidation of [1-^{14}C]pyruvate. Gaseous $^{14}CO_2$ was trapped in benzethonium hydroxide for 60 min and placed in a scintillation vial containing EcoLite liquid scintillation cocktail (MP Biomedicals, Santa Ana, CA) and counted. ^{14}C-labeled acid-soluble metabolites were measured by transfer aqueous phase after lipid extraction of incubated muscle into a scintillation vial and counted. Metabolic flexibility was assessed by comparing [1-^{14}C]pyruvate oxidation with and without 100 μM palmitic acid. The degree to which pyruvate oxidation decreased in the presence of free fatty acid (FFA) was used as an index of metabolic flexibility. A greater reduction (%decrease) in pyruvate oxidation in the presence of palmitate is indicative of appropriate substrate switching and thus metabolic flexibility (47).

Enzyme activity. Muscle samples were homogenized using a homogenizing buffer containing 0.175 mM KCl and 2.0 mM EDTA (pH = 7.4). Citrate synthase and β-hydroxyacyl-CoA dehydrogenase (β-HAD) activities were determined spectrophotometrically, as previously described (25).

Analysis of mRNA abundance. Analysis of gene expression was performed as described previously (43). Briefly, total RNA was isolated from ~50 mg of tissue using TRIzol reagent (5 Prime, Gaithersburg, MD) and purified using the Qiagen (Germantown, MD) RNeasy Mini Kit with on-column DNase digestion to remove genomic DNA contamination. Quantity and quality of RNA were assessed by absorbance at 260 nm and verified using the Experion System (Bio-Rad Laboratories, Hercules, CA). Total RNA (1,000 ng) was reverse-transcribed using iScript reverse transcriptase (Bio-Rad Laboratories). Real-time SYBR green PCR assays were performed with porcine-specific primers (Table 1), including carnitine palmitoyl transferse 1 (CPT-1), acetyl-CoA carboxylase (ACC)1, ACC2, and β-HAD, related to fatty acid metabolism. PCR reactions were performed in a 20-μl volume using SsoAdvanced universal SYBR Green Supermix (Bio-Rad Laboratories). Reactions contained a 1-μl primer set and diluted cDNA (10 ng). PCR quantification of each sample was performed in triplicate, and SYBR Green fluorescence was quantified with the CFX96 Touch Real Time PCR Detection System (Bio-Rad Laboratories). For

Table 1. *Primers used for qPCR*

Target Name	Sequence (5′to 3′)	Amplicon Size, bp
CPT-1		
Forward	AGGCCTCATCAAGAAGTGCC	183
Reverse	TGAACGAAGGCTGTGGACTC	
ACC1		
Forward	CTGGAGGTGTATGTGCGAAG	177
Reverse	GTGGTTGAGGTTGGAGGAGA	
ACC2		
Forward	TCATCCGCGTCATCATCAGG	127
Reverse	GTGTTGTTGAACGCCACCTC	
β-HAD		
Forward	GCTGGACAAGTTTGCTGCTG	116
Reverse	AGAAATGGAGCCCGGCAAAT	
18 s		
Forward	GTAACCCGTTGAACCCCAT	151
Reverse	CCATCCAATCGGTAGTAGCG	

ACC1, acetyl-CoA carboxylase-1; ACC2, acetyl-CoA carboxylase-2; β-HAD, β-hydroxyacyl-CoA dehydrogenase; CPT-1, carnitine palmitoyltransferase-1.

each assay, 40 PCR cycles were run, and a dissociation curve was included to verify the amplification of a single PCR product. Amplicons were sequenced to verify primer specificity. Analyses of amplification plots were performed with the CFX Manager Software version (Bio-Rad Laboratories). Data were analyzed using a five-point standard curve generated using serial 10-fold dilutions of TOPO-TA plasmid (Invitrogen) inserted with target genes. Each assay plate contained negative controls and a standard curve to determine amplification efficiency of the respective primer pair. Unknown sample expression was then determined from the standard curve and normalized for 18S expression.

Western immunoblot analysis. Total cellular protein extracts were prepared as described previously (16). Briefly, 50 mg of muscle biopsy were homogenized in lysis buffer (10 mM Tris·HCl, 1 mM EGTA, 150 mM NaCl, and 1% Triton X-100) containing 1× protease and phosphatase inhibitor cocktail (Thermo Scientific) using tissue lyser (Thermo Scientific). The whole tissue lysates were centrifuged at 13,000 *g* for 15 min at 4°C, and the supernatant was collected. Protein content of extracts was measured with the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Fixed protein amounts were electrophoresed using 10% SDS PAGE for CPT-1, ACC1, and α-tubulin and 6% SDS PAGE for ACC2 (ACC2 was separated from ACC1 and detected by total ACC antibody) and then transferred electrophoretically onto nitrocellulose membranes (Bio-Rad Laboratories). The membranes were blocked and incubated with primary antibodies validated for porcine extract in competition experiments with the blocking peptide for each antibody. Membranes were immunoblotted with primary antibodies against α-tubulin (1:1,000; Cell Signaling Technology), CPT-1 (1:1,000; Bioscience), ACC1 (1:1,000; Cell Signaling Technology), phospho-ACC (1:2,000, cell signaling), and total ACC (1:1,000; Cell Signaling Technology), followed by anti-rabbit secondary antibodies (1:20,000; Li-Cor). Signals were detected and quantified by densitometry using the Li-Cor Odyssey imaging system.

Statistical analysis. Data were analyzed by SAS (Cary, NC). The body weight, daily FI, average daily gain, body temperature, and respiratory rate were analyzed using the Proc Mixed procedure. The remainder of the data was analyzed using the Proc Mixed procedure with repeated measurements and using *day −2* data as a covariate. A pig was included in the model as a random variable. The model included treatment (TN, HS, and PF), day (3, 7), and the treatment-by-day interaction. Data are reported as least square means ± SE and are considered significant if $P < 0.05$.

RESULTS

To confirm that the heat load protocol caused heat stress in the pigs, physiological and metabolic indices were measured. Pigs exposed to HS had higher body temperatures ($P < 0.01$) compared with thermal neutral and pair-fed pigs throughout the experiment (Fig. 1*A*). Pair-fed pigs had a lower body temperature from *day 3* of the experiment compared with TN and HS pigs ($P < 0.05$), and this difference in body temperature was maintained until the end of the experiment. The average daily feed intake (ADFI) in TN pigs was 1.5 kg. Feed intake was decreased immediately in HS pigs on *day 1* and remained depressed throughout the experiment, as ADFI was 0.99 kg ($P < 0.01$; Fig. 1*B*). Pair-fed controls consumed an amount of feed similar (ADFI: 0.98 kg) to that of the HS pigs. The respiration rate was significantly increased in HS pigs through *day 1* to *day 7* ($P < 0.01$; Fig. 1*C*). At the end of the experiment, TN pigs had increased body weight (41.5 kg) compared with PF (37.8 kg) and HS (37.1 kg) pigs ($P < 0.05$; Fig. 1*D*). Average daily gain was decreased by >70% in PF and HS pigs throughout the experiment ($P < 0.01$; Fig. 1*E*).

Plasma glucose, insulin, and protein values are presented in Table 2. When compared with *day 7*, all of the groups tended to have higher blood glucose on *day 3* ($P = 0.09$). Total proteins, albumin, and globulin were not affected by any of the treatments. Aspartate aminotransferase (AST) increased by 54% in the HS pigs compared with PF pigs, and the AST level in TN pigs did not differ from either HS or PF pigs ($P < 0.05$). γ-Glutamyltransferase (GGT) was higher in PF pigs compared with TN and HS pigs ($P < 0.01$; Table 2) but did not differ between TN and HS pigs.

Next, we sought to determine the effect of heat stress and reduced plane of nutrition on the ability of skeletal muscle to metabolize different fuel substrates. The complete fatty acid oxidation measured as CO_2 production was decreased in HS pigs ($P < 0.01$; Fig. 2*A*). The incomplete fatty acid oxidation measured as acid-soluble metabolites (ASM) was also lower in HS pigs ($P < 0.01$; Fig. 2*B*). There was a treatment difference in total fatty acid oxidation ($P < 0.01$; Fig. 2*C*), as it was higher in the pair-fed pig compared with HS pigs (PF vs. HS $P = 0.01$) and tended to be higher than TN pigs (PF vs. TN $P = 0.1$). There were no environmental differences in fatty acid oxidation efficiency (CO_2/ASM; Fig. 2*D*). Glucose oxidation was decreased by HS ($P < 0.05$; Fig. 2*E*). The metabolic flexibility was increased from *day 3* to *day 7* of the experiment ($P < 0.01$). Heat stress inhibited metabolic flexibility by ~40 and 55% compared with TN and PF, respectively ($P < 0.05$; Fig. 2*F*).

There was an interaction between day and treatments in phosphofructokinase (PFK) activity ($P < 0.05$), as PFK activity was increased in PF pigs compared with TN pigs on *day 7* ($P < 0.05$; Fig. 3*A*), but PFK activity in HS pigs did not differ from either TN or PF pigs (Fig. 3*A*). The overall PDH activity was increased on *day 7* ($P < 0.01$; Fig. 3*B*,), and there was also a treatment difference ($P < 0.05$). The activity of PDH was increased in PF pigs compared with TN and HS pigs ($P < 0.05$), and no difference was detected between TN and HS pigs. Citrate synthase activity was decreased in HS pigs compared with TN controls ($P < 0.05$; Fig. 3*C*), but PF pigs did not differ from TN or HS pigs. There was a day-by-treatment interaction in β-HAD activity ($P < 0.05$; Fig. 3*D*). On *day 7*,

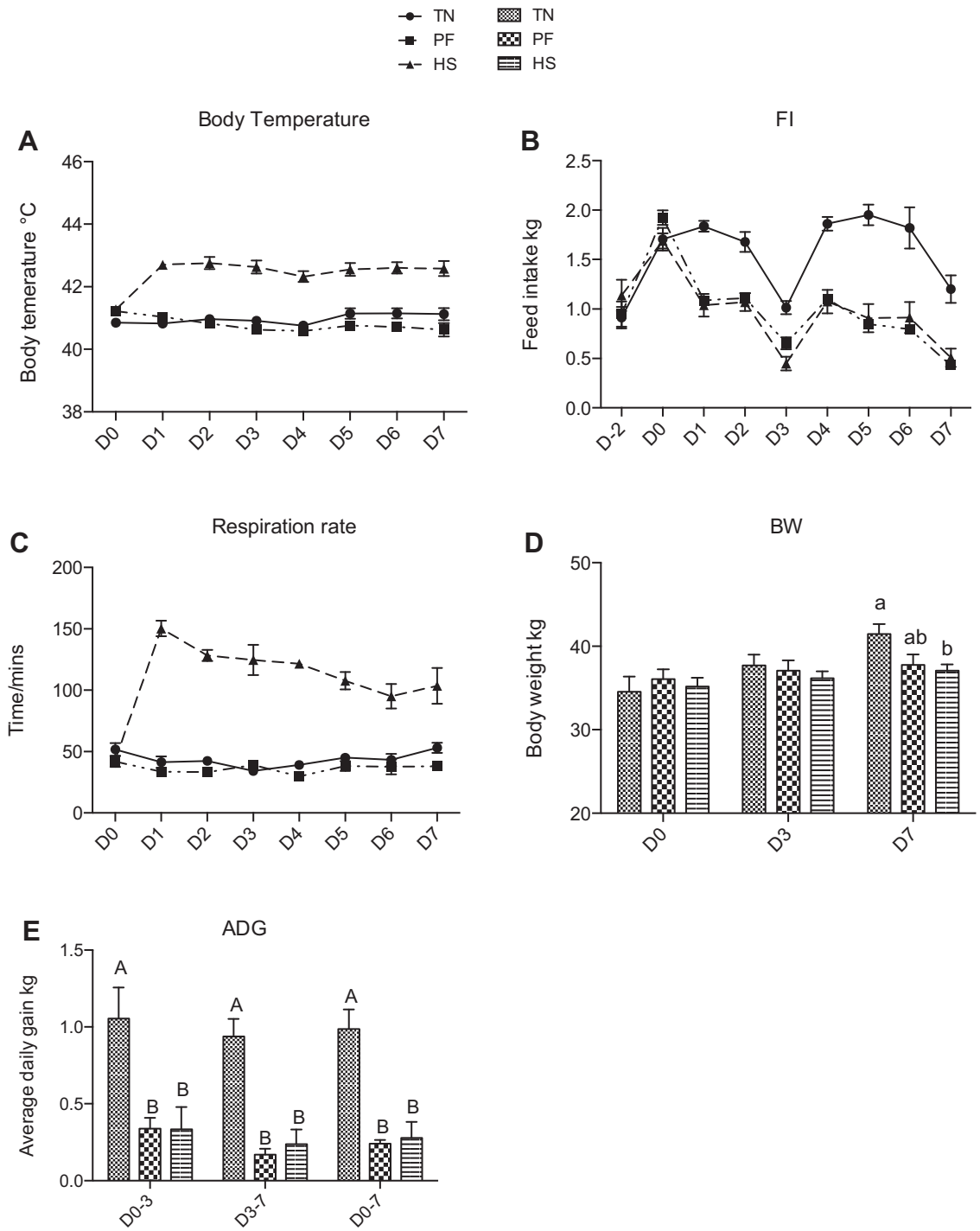

Fig. 1. Effects of heat stress on body temperature (*A*), feed intake (FI; *B*), respiration rate (*C*), body weight (BW; *D*), and average daily gain (ADG; *E*) in growing pigs. Pigs were exposed to thermal neutral (TN), pair-fed (PF), or heat stress (HS) conditions for 7 days. Body temperature and feed intake were measured daily. Body weights were measured on *days 0* (D0), *3* (D3), and *7* (D7). Results are presented as means ± SE; $n = 8$ pigs. Different letters indicate significance within day: lowercase letters, $P < 0.05$; uppercase letters, $P < 0.01$. D−2, *day −2*.

β-HAD activity was decreased in HS pigs compared with TN pigs ($P < 0.05$), and PF pigs did not differ from TN or HS pigs.

Finally, we sought to examine the molecular basis for the apparently altered fuel substrate metabolism in heat-stressed skeletal muscle. Carnitine palmitoyltransferase-1 (CPT-1) mRNA expression was greater in PF pigs compared with TN or HS ($P < 0.05$; Fig. 4*A*). On *day 3*, CPT-1 mRNA tended to be decreased in HS pigs ($P = 0.08$). There was a day-by-treatment interaction in ACC1 mRNA expression as PF and HS pigs expressed <30% ACC1 of TN pigs ($P < 0.05$; Fig. 4*B*). On *day 3*, there was a decrease in ACC2 mRNA abundance in HS pigs compared with TN ($P < 0.05$; Fig. 4*C*). There tended to be a day-by-treatment interaction in β-HAD mRNA expression, as it was decreased in HS compared with TN pigs on *day*

Table 2. *Effect of heat stress on circulating metabolic parameters in pigs*

	Treatment				*P* Value		
Metabolic Parameters (day)	TN	PF	HS	SE	Treatment	Day	Interaction
Glucose, mg/dl							
Day −2		114.1	115.5	6.06	0.56	0.09	0.37
Day 3	96.5	108.8	98.9				
Day 7	85.3	102.6	92.1				
Insulin, μg/l							
Day −2	0.091	0.048	0.069	0.02	0.33	0.62	0.54
Day 3	0.060	0.052	0.038				
Day 7	0.028	0.031	0.069				
G:I ($\times 10^7$)							
Day −2	2.27	5.67	4.06	2.10	0.42	0.68	0.74
Day 3	3.84	4.45	6.81				
Day 7	4.38	6.73	2.62				
Total protein, g/dl							
Day 0	6.00	5.88	5.76	0.12	0.28	0.03	0.82
Day 3	6.11	6.22	5.95				
Day 7	6.39	6.18	6.34				
Albumin, g/dl							
Day −2	3.23	3.16	3.08	0.08	0.25	0.34	0.89
Day 3	3.46	3.58	3.40				
Day 7	3.19	3.55	3.57				
Globulin, g/dl							
Day −2	2.77	2.73	2.70	0.10	0.98	0.09	0.61
Day 3	2.50	2.62	2.56				
Day 7	2.61	2.62	2.52				
AST, U/l							
Day −2	39.00	29.25	37.87	3.18	0.01	0.02	0.04
Day 3	28.50	21.50	45.13				
Day 7	30.56	22.61	32.13				
GGT, U/l							
Day −2	25.13	23.38	26.00	2.11	<0.01	0.09	0.86
Day 3	22.78	25.25	24.63				
Day 7	24.53	28.58	24.41				

AST, aspartate aminotransferase; GGT, γ-glutamyltransferase; G:I, glucose-to-insulin ratio; HS, heat stress; PF, pair fed; TN, thermal neutral.

3 and *day 7* ($P < 0.08$; Fig. 4*D*). There was no difference in CPT-1 protein abundance among the treatments (Fig. 5*A*). Total ACC protein abundance in HS was decreased on *day 3* and *day 7* (~30%), although there was no statistical significance (Fig. 5*B*). Consistent with mRNA data, there was a day-by-treatment interaction in ACC1 protein abundance ($P < 0.05$; Fig. 5*C*). The abundance of ACC1 protein was significantly lower in HS and PF pigs on *day 7* compared with TN pigs. ACC2 protein abundance was unchanged (Fig. 5*D*). There was a tendency in day-by-treatment interaction in phosphorylation of ACC protein abundance ($P < 0.07$; Fig. 5*E*)

DISCUSSION

An immediate effect of HS is decreased feed intake and increased respiratory rate, which may minimize metabolic heat production and maximize heat dissipation, respectively. Studies have shown that nutrient restriction was not the only driver of altered metabolism during heat stress, as heat stress directly affects metabolism (11, 39, 50). Therefore, we used TN ad libitum and PF (nutrient restricted) in the TN condition as controls to elucidate the direct and indirect effects of HS on metabolism. Although the PF model is likely the best model to demonstrate that the effect of HS on metabolism is related/unrelated to plane of nutrition, it is important to note that the PF pigs may represent a different feeding pattern compared with the HS pigs. The PF pigs were given two meals a day; however, the HS pigs may consume smaller meals on a different schedule each day (8). Thus, the results described herein are specific to the experimental protocol and feeding regimen described. Additional studies could investigate the effects of feeding pattern during heat stress and reduced plane of nutrition on metabolic outcomes.

In the current study, application of the heat load was sufficient to cause heat stress, as the HS pigs displayed elevated body temperature and respiratory rate throughout the experiment, which has been widely observed in both chronic and acute heat stress animals (20, 38, 48). Consistent with a previous study (39), PF pigs had lower body temperature after *day 3* compared with TN, which presumably was caused by reduced thermic effect of feeding (the heat associated with digesting, absorbing, and assimilating nutrients). It has been reported that the body temperature increases then decline over time of exposure to high ambient temperature, which indicates the acclimation to chronic heat stress (42). However, we did not observe such responses. Some variables in this study, such as the magnitude of heat stress, genetics of the pigs, etc., may be sufficiently different from other studies to prevent or delay an adaptation response.

Several biochemical changes are associated with heat stress. An increase in the plasma AST was observed in HS pigs, and this corroborates findings in poultry and humans (2, 31). AST is used as a clinical marker of liver function in heat stroke patients with an elevation consistent with liver injury (37). Our

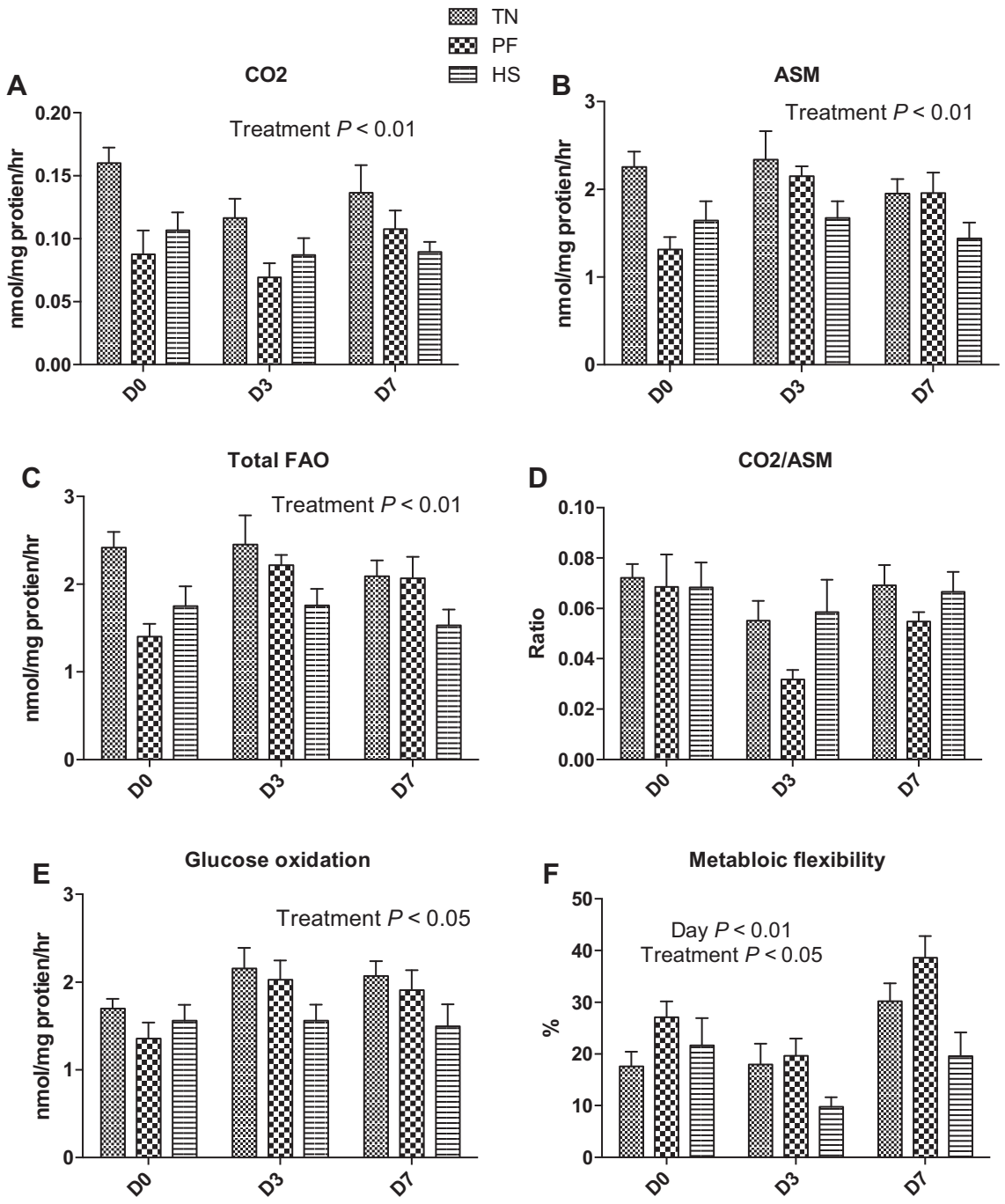

Fig. 2. Effects of heat stress on fatty acid oxidation (FAO) in the Longissimus dorsi (LD) muscle of growing pigs. *A*: complete FAO. *B*: incomplete FAO. *C*: total fatty acid oxidation. *D*: oxidation efficiency. *E*: glucose oxidation. *F*: metabolic flexibility. Pigs were exposed to thermal neutral (TN), pair-fed (PF), or heat stress (HS) conditions for 7 days. LD biopsies were taken before the experiment and on *day 3* (D3) and *day 7* (D7) heat stress. Results are presented as means ± SE; $n = 8$ pigs. ASM, acid-soluble metabolites. D0, *day 0*.

data indicate that heat stress may cause liver dysfunction in pigs.

The decreased basal glucose concentration by heat stress was found in multiple animal models, including rodents, dogs, and sheep (1, 29, 36). In this study, we did not observe a significant difference in the fasting blood glucose concentration among treatments, which is consistent with our previous study (50). By *day 7*, HS pigs numerically increased plasma insulin concentration by 50% compared with PF pigs, but there was no statistical significance. However, other studies reported that HS increased secretion of insulin in pigs, cattle, and mice (6, 11, 50).

Heat stress alters overall metabolic rate (3, 7, 22), although energy demands within individual body tissues will vary depending on the extent of damage sustained or the function of that tissue. Skeletal muscle, by virtue of its contribution to body mass, can dramatically influence whole body metabolism by altering fuel substrate dynamics. Shifting metabolic flexi-

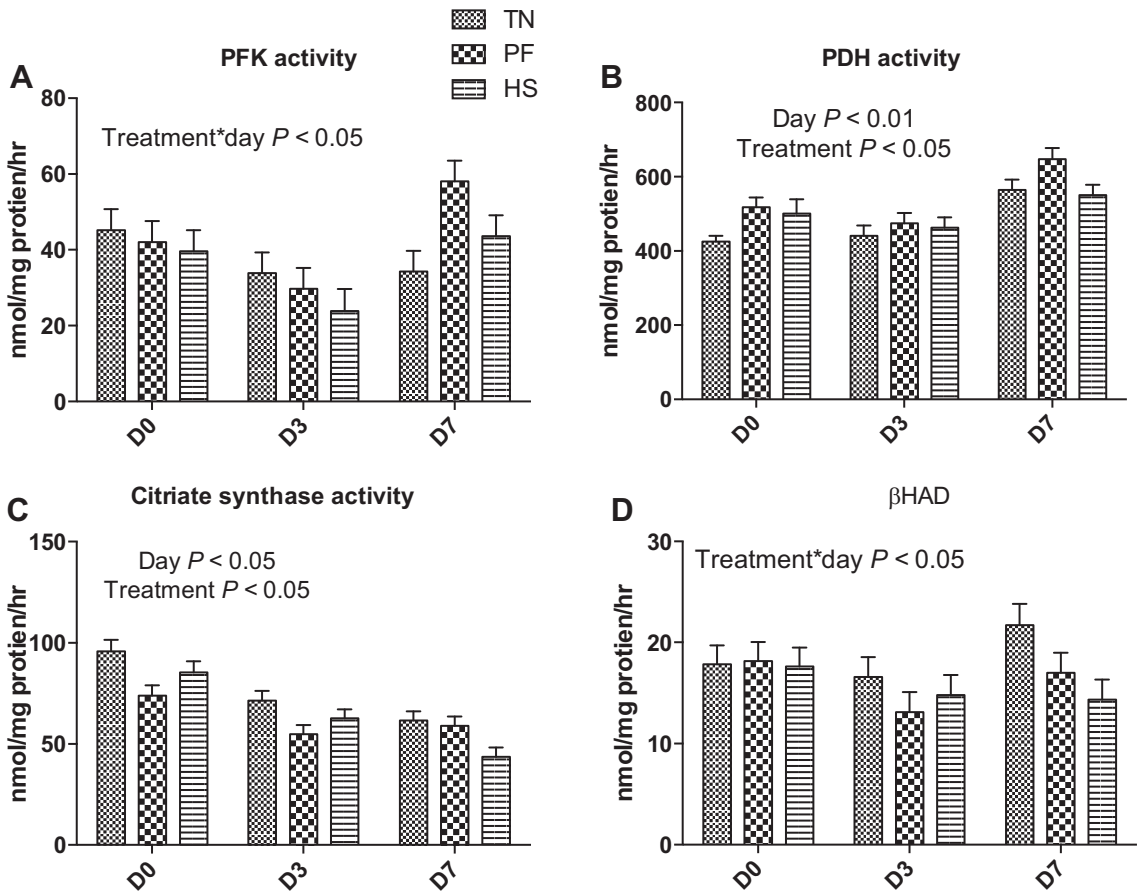

Fig. 3. Effects of heat stress on enzyme activities in the Longissimus dorsi (LD) muscle of growing pigs. *A*: phosphofructokinase (PKF) activity. *B*: pyruvate dehydrogenase (PDH) kinase activity. *C*: citrate synthase activity. *D*: β-hydroxyacyl-CoA dehydrogenase (β-HAD) activity. Pigs were exposed to thermal neutral (TN), pair-fed (PF), or heat stress (HS) conditions for 7 days. LD biopsies were taken before the experiment and on *day 3* (D3) and *day 7* (D7) heat stress. Results are presented as means ± SE; $n = 8$ pigs. Different letters indicate significance at $P < 0.05$. D0, *day 0*.

bility within skeletal muscle and/or changing the metabolic products exported for use in interorgan metabolic pathways underlies such influences. Indeed, evidence indicates that skeletal muscle fuel selection appears to favor carbohydrate use at the expense of lipid (12). Studies showed increased reliance on intramuscular carbohydrates during exercise in the heat (23, 27), whereas heat during exercises decreased muscle carbohydrate oxidation (13, 14). In agreement, the current study demonstrated that glucose oxidation rate was reduced by heat stress. Sano et al. (44) found that HS decreased glucose turnover rate but did not change the proportion of glucose oxidized in sheep in vivo. Taken together, further studies are needed to investigate altered glucose and insulin dynamics during HS.

Feed-restricted animals use free fatty acids (FFA) from adipose tissue to spare glucose for skeletal muscle deposition (6). To test whether heat stress affects FFA utilization in muscle, we measured the fatty acid oxidation in the longissimus dorsi muscle and found that HS pigs had similar total fatty acid oxidation rate as TN pigs and a significantly lower fatty acid oxidation rate compared with PF pigs. Additionally, the incomplete fatty acid oxidation tended to be decreased in HS pigs. The FFA concentrations during HS and feed restriction conditions has been studied widely in both swine and ruminants (6, 7, 50). However, there is no study that compares the utilization of FFA in muscle among TN, HS, and PF conditions. It has been reported that plasma fatty acid oxidation is primarily determined by the fatty acid concentration. Our fatty acid oxidation data are consistent with FFA concentration in TN, HS, and PF pigs. Under similar nutrient conditions, PF pigs mobilize adipose tissue to a greater extent, as they had higher plasma FFA, whereas HS pigs had similar FFA concentration as TN pigs (39). Decreased FFA concentrations in hyperthermic conditions compared with the PF treatment were also shown in sheep and cattle (11, 44, 46). Additionally, in PF pigs (feed restricted), FFA are the predominant fuel used by skeletal muscle (39). Therefore, PF pigs had higher total fatty acid oxidation than TN and HS pigs. These data also indicate that, despite the low plane of nutrition, fatty acid utilization was blunted by heat stress.

Based on the Randle cycle, there is competition between fatty acid oxidation and glucose oxidation (40). Artificially increasing fatty acid oxidation by adding FFA decreases glucose oxidation (30). In this study, metabolic flexibility was assessed by measuring pyruvate oxidation with and without the presence of FFA, and the percentage decrease in pyruvate oxidation in the presence of FFA was calculated. Our current results show that heat stress decreased mitochondrial metabolic flexibility, further substantiating our findings that HS pigs had limited ability to use FFA. Pair-fed pigs, on a reduced plane of

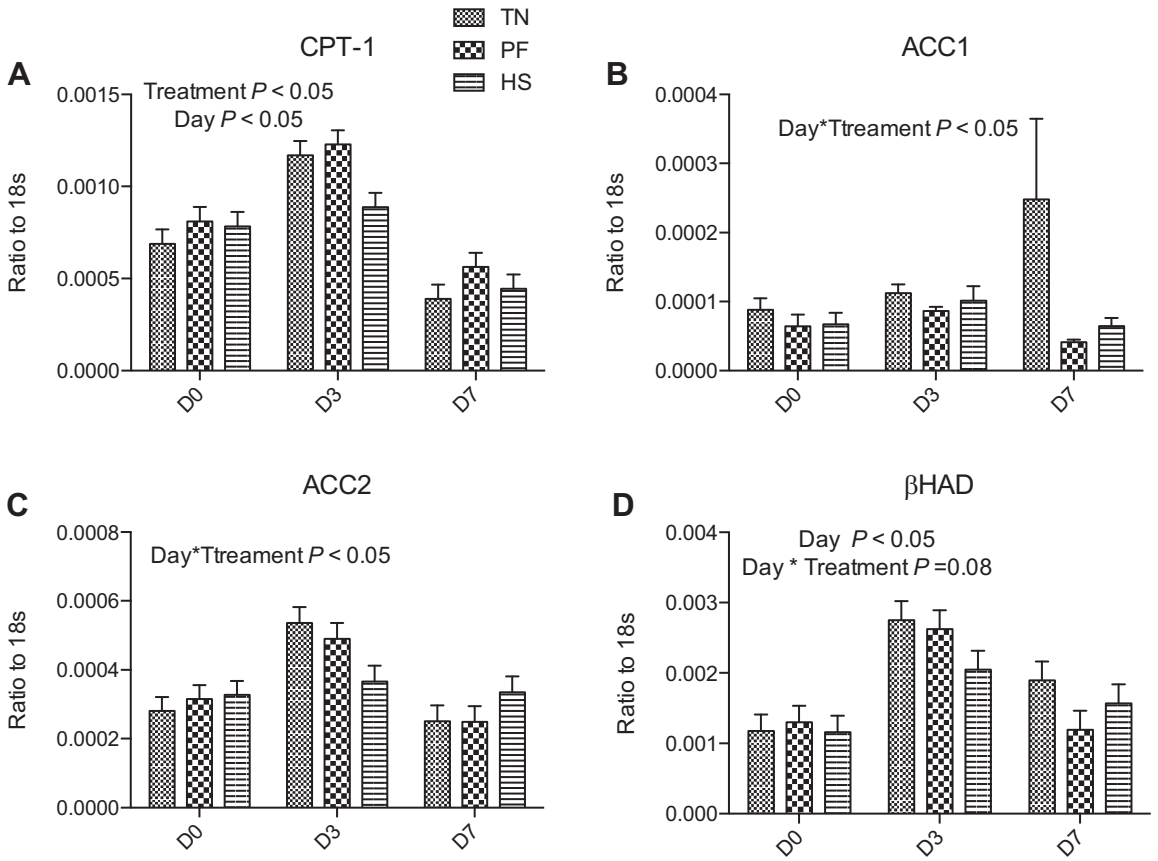

Fig. 4. Effects of heat stress on carnitine palmitoyltransferase-1 (CPT-1; *A*), acetyl-CoA carboxylase-1 (ACC1; *B*), acetyl-CoA carboxylase-2 (ACC2; *C*), and β-hydroxyacyl-CoA dehydrogenase (β-HAD) mRNA expression (*D*) in the Longissimus dorsi (LD) muscle of growing pigs. Pigs were exposed to thermal neutral (TN), pair-fed (PF), or heat stress (HS) conditions for 7 days. LD biopsies were taken before the experiment and on on *day 3* (D3) and *day 7* (D7) heat stress. Results are presented as means ± SE; $n = 8$ pigs. Different letters indicate significance at $P < 0.05$. D0, *day 0*.

nutrition, had similar metabolic flexibility as the TN controls. Our present findings provide evidence that skeletal muscle in HS pigs may not use fatty acids as the primary fuel source. Therefore, previous reports indicate that the mobilization of FFA from adipose tissue is inhibited during heat stress (discussed above), and the capacity to utilize fatty acids in skeletal muscle shown herein also appears to be attenuated. Consequences of heat stress include reduced feed intake and shunted blood flow away from the gastrointestinal tract (GIT) causing damage and compromised integrity of the GIT (33, 38). The resulting "leaky gut" allows infiltration of endotoxin into the circulation to elicit effects on various tissues, including liver and skeletal muscle. The ability of endotoxin to directly affect tissue responses is mediated by activation of the Toll-like 4 receptor (TLR4) (18, 19). In skeletal muscle, Frisard et al. (17) demonstrated that activation of TLR4 leads to altered metabolism, including reduced metabolic flexibility and fatty acid oxidation, similar to our heat stress responses presented herein. Thus, the reduced capacity to rely on fatty acids as a fuel may be the consequence of heat-induced gastrointestinal hyperpermeability.

To further elucidate how HS changed metabolic flexibility, the activities of the key enzymes in glucose oxidation and fatty acid oxidation were measured. Phosphofructokinase, the rate-limiting enzyme in glycolysis, was increased in PF compared with TN pigs, indicating increased glycolysis. Increased glycolysis may produce more pyruvate, which positively regulates PDH. As a result, PF pigs also had increased PDH activity. Glycolysis in HS pigs did not differ from TN or PF pigs. However, both citrate synthase activity and β-HAD activity were inhibited by heat stress, suggesting decreased oxidative capacity. Citrate synthase activity, a biomarker of mitochondrial content, is also associated with morphological change in mitochondrial content (34, 41). Our previous studies examining the acute effect of heat stress on skeletal muscle demonstrate altered inflammatory signaling, redox balance, and dysfunctional autophagy, which may cause immediate and persistent skeletal muscle dysfunction (20, 48). The aforementioned changes could directly impinge upon mitochondrial function, producing altered cellular metabolism during both acute and chronic heat stress. Hence, it is reasonable to suggest that heat stress may change metabolic flexibility via alteration of mitochondrial function or content or modifying mitochondrial morphology.

We also measured CPT-1, the enzyme responsible for the transfer of long-chain free fatty acids into the mitochondria, to test whether decreased fatty acid oxidation by heat stress is due to decreased transportation. CPT-1 mRNA expression tended to decrease in heat-stressed pigs; however, protein abundance was similar between groups. Acetyl-CoA is the intermediate product in carbohydrate, amino acid, and fatty acid metabolism. Fatty acid synthesis requires malonyl-CoA as

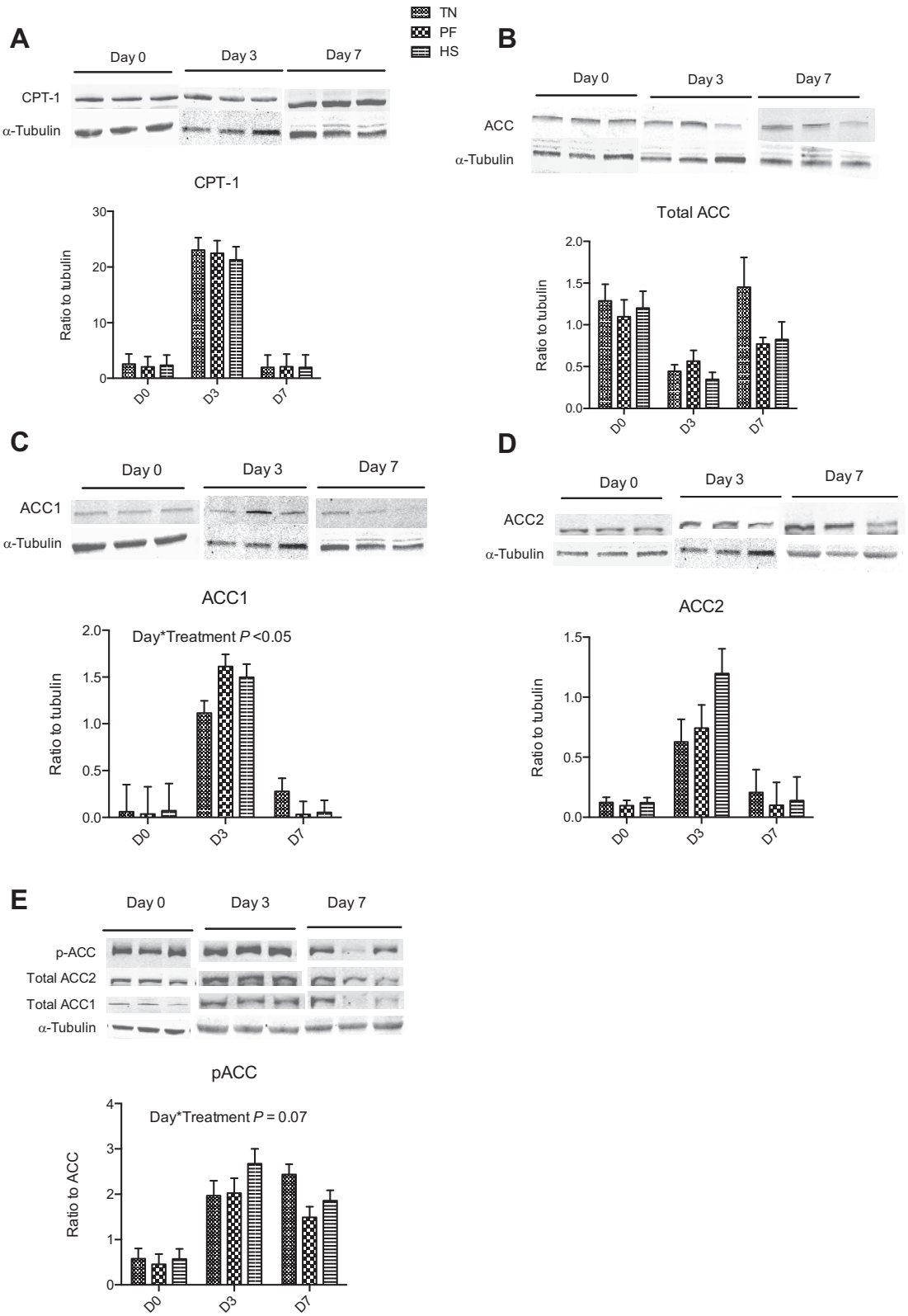

Fig. 5. Effects of heat stress on carnitine palmitoyltransferase-1 (CPT-1; *A*), total acetyl CoA carboxylase (ACC; *B*), acetyl-CoA carboxylase-1 (ACC1; *C*), acetyl-CoA carboxylase-2 (ACC2; *D*) and phospho-acetyl-CoA carboxylase protein (pACC; *E*) abundance in the Longissimus dorsi (LD) muscle of growing pigs. Pigs were exposed to thermal neutral (TN), pair-fed (PF), or heat stress (HS) conditions for 7 days. LD biopsies were taken before the experiment and on *day 3* (D3) and *day 7* (D7) heat stress. Results are presented as means ± SE; $n = 8$ pigs. Different letters indicate significance at $P < 0.05$. D0, *day 0*.

the C_2 donor, which is formed by acetyl-CoA catalyzed by ACC. Malonyl-CoA is not only the building block of long chain fatty acid but is also a regulator of the carnitine palmitoyl-CoA shuttle system that is involved in fatty acid oxidation. Two isoforms of ACC with different functions have been detected: ACC1 and ACC2 (35). ACC1-generated malonyl-CoA is used by fatty acid synthesis; however, ACC2-generated malonyl-CoA is the inhibitor of CPT-1 (49). In the present study, although ACC2 mRNA expression was inhibited by heat stress on *day 3*, protein abundance was similar between groups. Taken together with CPT-1 data, heat stress may not inhibit fatty acid uptake in Longissimus dorsi muscle. On the other hand, ACC1 decreased at both mRNA and protein levels in PF and HS pigs. We also measured the total phosphorylation of ACC. Phosphorylated ACC tended to be decreased in PF and HS pigs, which suggests increased fatty acid synthesis in Longissimus dorsi muscle. Both PF and HS pigs had higher fatty acid synthesis, whereas HS pigs also had lower fatty acid oxidation rate. These findings may partially explain adipose tissue accumulation during heat stress.

Perspectives and Significance

In summary, pigs exposed to heat had higher body temperature, lower body weight, and lower average daily gain compared with TN pigs as expected. Compared with PF pigs, the ability of skeletal muscle to oxidize fatty acids was markedly decreased during heat stress conditions. Concomitantly, heat stress conditions decreased metabolic flexibility and oxidative enzyme activity. These results were caused by direct effects of HS on metabolism independent of lower plane of nutrition (an indirect effect of HS). Together, these results indicate that heat stress alters the metabolic fuel priorities within skeletal muscle regardless of plane of nutrition. Future studies are needed to determine the impact of the altered metabolism on skeletal muscle performance during heat conditions.

GRANTS

This work was supported by United States Department of Agriculture/Agriculture and Food Research Initiative Grants 2014-67015-21627 and 2011-6700330007.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L.D.Z., G.X., S.G.L.W.G., L.H.B., S.W.E.-K., J.T.S., J.W.R., N.K.G., and R.P.R. conceived and designed research; L.D.Z., R.P.M., G.X., S.G.L.W.G., and R.P.R. performed experiments; L.D.Z., R.P.M., L.H.B., S.W.E.-K., M.W.H., and R.P.R. analyzed data; L.D.Z., R.P.M., L.H.B., S.W.E.-K., J.T.S., J.W.R., N.K.G., M.W.H., and R.P.R. interpreted results of experiments; L.D.Z. and R.P.R. prepared figures; L.D.Z. and R.P.R. drafted manuscript; L.D.Z., R.P.M., G.X., S.G.L.W.G., L.H.B., S.W.E.-K., J.T.S., J.W.R., N.K.G., M.W.H., and R.P.R. edited and revised manuscript; L.D.Z., R.P.M., G.X., S.G.L.W.G., L.H.B., S.W.E.-K., J.T.S., J.W.R., N.K.G., M.W.H., and R.P.R. approved final version of manuscript.

REFERENCES

1. **Achmadi J, Yanagisawa T, Sano H, Terashima Y.** Pancreatic insulin secretory response and insulin action in heat-exposed sheep given a concentrate or roughage diet. *Domest Anim Endocrinol* 10: 279–287, 1993. doi:10.1016/0739-7240(93)90032-7.
2. **Alzeer AH, el-Hazmi MA, Warsy AS, Ansari ZA, Yrkendi MS.** Serum enzymes in heat stroke: prognostic implication. *Clin Chem* 43: 1182–1187, 1997.
3. **Ames DR, Nellor JE, Adams T.** Energy balance during heat stress in sheep. *J Anim Sci* 32: 784–788, 1971. doi:10.2527/jas1971.324784x.
4. **Bauman DE, Currie WB.** Partitioning of nutrients during pregnancy and lactation: a review of mechanisms involving homeostasis and homeorhesis. *J Dairy Sci* 63: 1514–1529, 1980. doi:10.3168/jds.S0022-0302(80)83111-0.
5. **Baumgard LH, Rhoads RP.** Ruminant Nutrition Symposium: ruminant production and metabolic responses to heat stress. *J Anim Sci* 90: 1855–1865, 2012. doi:10.2527/jas.2011-4675.
6. **Baumgard LH, Rhoads RP Jr.** Effects of heat stress on postabsorptive metabolism and energetics. *Annu Rev Anim Biosci* 1: 311–337, 2013. doi:10.1146/annurev-animal-031412-103644.
7. **Blaxter K, Graham N, Wainman F, Armstrong D.** Environmental temperature, energy metabolism and heat regulation in sheep. I. Energy metabolism in closely clipped sheep. *J Agric Sci* 52: 16, 1959.
8. **Collin A, van Milgen J, Dubois S, Noblet J.** Effect of high temperature on feeding behaviour and heat production in group-housed young pigs. *Br J Nutr* 86: 63–70, 2001. doi:10.1079/BJN2001356.
9. **El-Kadi SW, Suryawan A, Gazzaneo MC, Srivastava N, Orellana RA, Nguyen HV, Lobley GE, Davis TA.** Anabolic signaling and protein deposition are enhanced by intermittent compared with continuous feeding in skeletal muscle of neonates. *Am J Physiol Endocrinol Metab* 302: E674–E686, 2012. doi:10.1152/ajpendo.00516.2011.
10. **Ellis FP.** Mortality from heat illness and heat-aggravated illness in the United States. *Environ Res* 5: 1–58, 1972. doi:10.1016/0013-9351(72)90019-9.
11. **Elsasser TH, Rhoads RP, Kahl S, Collier R, Baumgard LH, Li C, Caperna TJ.** Heat stress augments plasma tyrosine-nitrated proteins and lactate-to-pyruvate ratio after repeated endotoxin (LPS) challenge in steers. *J Anim Sci* 87: 9–16, 2009. doi:10.2527/jas.2008-1128.
12. **Febbraio MA.** Alterations in energy metabolism during exercise and heat stress. *Sports Med* 31: 47–59, 2001. doi:10.2165/00007256-200131010-00004.
13. **Febbraio MA, Snow RJ, Hargreaves M, Stathis CG, Martin IK, Carey MF.** Muscle metabolism during exercise and heat stress in trained men: effect of acclimation. *J Appl Physiol (1985)* 76: 589–597, 1994. doi:10.1152/jappl.1994.76.2.589.
14. **Febbraio MA, Snow RJ, Stathis CG, Hargreaves M, Carey MF.** Effect of heat stress on muscle energy metabolism during exercise. *J Appl Physiol (1985)* 77: 2827–2831, 1994. doi:10.1152/jappl.1994.77.6.2827.
15. **Fink WJ, Costill DL, Van Handel PJ.** Leg muscle metabolism during exercise in the heat and cold. *Eur J Appl Physiol Occup Physiol* 34: 183–190, 1975. doi:10.1007/BF00999931.
16. **Flann KL, Rathbone CR, Cole LC, Liu X, Allen RE, Rhoads RP.** Hypoxia simultaneously alters satellite cell-mediated angiogenesis and hepatocyte growth factor expression. *J Cell Physiol* 229: 572–579, 2014. doi:10.1002/jcp.24479.
17. **Frisard MI, McMillan RP, Marchand J, Wahlberg KA, Wu Y, Voelker KA, Heilbronn L, Haynie K, Muoio B, Li L, Hulver MW.** Toll-like receptor 4 modulates skeletal muscle substrate metabolism. *Am J Physiol Endocrinol Metab* 298: E988–E998, 2010. doi:10.1152/ajpendo.00307.2009.
18. **Frost RA, Nystrom GJ, Lang CH.** Lipopolysaccharide regulates proinflammatory cytokine expression in mouse myoblasts and skeletal muscle. *Am J Physiol Regul Integr Comp Physiol* 283: R698–R709, 2002. doi:10.1152/ajpregu.00039.2002.
19. **Frost RA, Nystrom GJ, Lang CH.** Lipopolysaccharide stimulates nitric oxide synthase-2 expression in murine skeletal muscle and C_2C1_{pigs} myoblasts via Toll-like receptor-4 and c-Jun NH_2-terminal kinase pathways. *Am J Physiol Cell Physiol* 287: C1605–C1615, 2004. doi:10.1152/ajpcell.00010.2004.
20. **Ganesan S, Volodina O, Pearce SC, Gabler NK, Baumgard LH, Rhoads RP, Selsby JT.** Acute heat stress activated inflammatory signaling in porcine oxidative skeletal muscle. *Physiol Rep* 5: e13397, 2017. doi:10.14814/phy2.13397.
21. **González-Alonso J, Calbet JA, Nielsen B.** Metabolic and thermodynamic responses to dehydration-induced reductions in muscle blood flow in exercising humans. *J Physiol* 520: 577–589, 1999. doi:10.1111/j.1469-7793.1999.00577.x.
22. **Graham NM, Wainman FW, Blaxter KL, Armstrong DG.** Environmental temperature, energy metabolism and heat regulation in sheep. I. Energy metabolism in closely clipped sheep. *J Agric Sci* 52: 13–24, 1959. doi:10.1017/S0021859600035632.

23. **Hargreaves M, Angus D, Howlett K, Conus NM, Febbraio M.** Effect of heat stress on glucose kinetics during exercise. *J Appl Physiol (1985)* 81: 1594–1597, 1996. doi:10.1152/jappl.1996.81.4.1594.
25. **Heilbronn LK, Civitarese AE, Bogacka I, Smith SR, Hulver M, Ravussin E.** Glucose tolerance and skeletal muscle gene expression in response to alternate day fasting. *Obes Res* 13: 574–581, 2005. doi:10.1038/oby.2005.61.
26. **Issad T, Pénicaud L, Ferré P, Kandé J, Baudon MA, Girard J.** Effects of fasting on tissue glucose utilization in conscious resting rats. Major glucose-sparing effect in working muscles. *Biochem J* 246: 241–244, 1987. doi:10.1042/bj2460241.
27. **Jentjens RLPG, Wagenmakers AJM, Jeukendrup AE.** Heat stress increases muscle glycogen use but reduces the oxidation of ingested carbohydrates during exercise. *J Appl Physiol (1985)* 92: 1562–1572, 2002. doi:10.1152/japplphysiol.00482.2001.
28. **Jeong JY, Jeoung NH, Park KG, Lee IK.** Transcriptional regulation of pyruvate dehydrogenase kinase. *Diabetes Metab J* 36: 328–335, 2012. doi:10.4093/dmj.2012.36.5.328.
29. **Kanter GS.** Effect of heat on regulation of body fluids and electrolytes in dogs. *Am J Physiol* 178: 259–262, 1954. doi:10.1152/ajplegacy.1954.178.2.259.
30. **Kelley DE, Mandarino LJ.** Fuel selection in human skeletal muscle in insulin resistance: a reexamination. *Diabetes* 49: 677–683, 2000. doi:10.2337/diabetes.49.5.677.
31. **Khan WA, Khan A, Anjum AD. Rehman Z.** Effects of induced heat stress on some biochemical values in broiler chicks. *Int J Agric Biol* 4: 2, 2002.
32. **Kozłowski S, Brzezińska Z, Kruk B, Kaciuba-Uściłko H, Greenleaf JE, Nazar K.** Exercise hyperthermia as a factor limiting physical performance: temperature effect on muscle metabolism. *J Appl Physiol (1985)* 59: 766–773, 1985. doi:10.1152/jappl.1985.59.3.766.
33. **Lambert GP.** Stress-induced gastrointestinal barrier dysfunction and its inflammatory effects. *J Anim Sci* 87, *Suppl* 14: E101–E108, 2009. doi:10.2527/jas.2008-1339.
34. **Larsen S, Nielsen J, Hansen CN, Nielsen LB, Wibrand F, Stride N, Schroder HD, Boushel R, Helge JW, Dela F, Hey-Mogensen M.** Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. *J Physiol* 590: 3349–3360, 2012. doi:10.1113/jphysiol.2012.230185.
35. **McGarry JD, Brown NF.** The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. *Eur J Biochem* 244: 1–14, 1997. doi:10.1111/j.1432-1033.1997.00001.x.
36. **Mitev S, Dinevska-Kovkarovska S, Miova B.** Effect of the acclimation to high environmental temperature on the activity of hepatic glycogen phosphorylase (a+b and a), liver glycogen content and blood glucose level in rats. *J Therm Biol* 30: 563–568, 2005. doi:10.1016/j.jtherbio.2005.07.003.
37. **Park GJH, Lin BPC, Ngu MC, Jones DB, Katelaris PH.** Aspartate aminotransferase: alanine aminotransferase ratio in chronic hepatitis C infection: is it a useful predictor of cirrhosis? *J Gastroenterol Hepatol* 15: 386–390, 2000. doi:10.1046/j.1440-1746.2000.02172.x.
38. **Pearce S.** *The Effects of Heat Stress and Nutritional Status on Metabolism and Intestinal Integrity in Growing Pigs*. Ames, IA: Iowa State University, 2011. doi:10.31274/etd-180810-2240.
39. **Pearce SC, Gabler NK, Ross JW, Escobar J, Patience JF, Rhoads RP, Baumgard LH.** The effects of heat stress and plane of nutrition on metabolism in growing pigs. *J Anim Sci* 91: 2108–2118, 2013. doi:10.2527/jas.2012-5738.
40. **Randle PJ.** Regulatory interactions between lipids and carbohydrates: the glucose fatty acid cycle after 35 years. *Diabetes Metab Rev* 14: 263–283, 1998. doi:10.1002/(SICI)1099-0895(199812)14:4<263::AID-DMR233>3.0.CO;2-C.
41. **Reichmann H, Hoppeler H, Mathieu-Costello O, von Bergen F, Pette D.** Biochemical and ultrastructural changes of skeletal muscle mitochondria after chronic electrical stimulation in rabbits. *Pflugers Arch* 404: 1–9, 1985. doi:10.1007/BF00581484.
42. **Renaudeau D, Kerdoncuff M, Anaïs C, Gourdine JL.** Effect of temperature level on thermal acclimation in Large White growing pigs. *Animal* 2: 1619–1626, 2008. doi:10.1017/S1751731108002814.
43. **Rhoads RP, Flann KL, Cardinal TR, Rathbone CR, Liu X, Allen RE.** Satellite cells isolated from aged or dystrophic muscle exhibit a reduced capacity to promote angiogenesis in vitro. *Biochem Biophys Res Commun* 440: 399–404, 2013. doi:10.1016/j.bbrc.2013.09.085.
44. **Sano H, Takahashi K, Ambo K, Tsuda T.** Turnover and oxidation rates of blood glucose and heat production in sheep exposed to heat. *J Dairy Sci* 66: 856–861, 1983. doi:10.3168/jds.S0022-0302(83)81867-0.
45. **Schwartz J.** Who is sensitive to extremes of temperature? A case-only analysis. *Epidemiology* 16: 67–72, 2005. doi:10.1097/01.ede.0000147114.25957.71.
46. **Shwartz G, Rhoads ML, VanBaale MJ, Rhoads RP, Baumgard LH.** Effects of a supplemental yeast culture on heat-stressed lactating Holstein cows. *J Dairy Sci* 92: 935–942, 2009. doi:10.3168/jds.2008-1496.
47. **Tarpey MD, Davy KP, McMillan RP, Bowser SM, Halliday TM, Boutagy NE, Davy BM, Frisard MI, Hulver MW.** Skeletal muscle autophagy and mitophagy in endurance-trained runners before and after a high-fat meal. *Mol Metab* 6: 1597–1609, 2017. doi:10.1016/j.molmet.2017.10.006.
48. **Volodina O, Ganesan S, Pearce SC, Gabler NK, Baumgard LH, Rhoads RP, Selsby JT.** Short-term heat stress alters redox balance in porcine skeletal muscle. *Physiol Rep* 5: e13267, 2017. doi:10.14814/phy2.13267.
49. **Wakil SJ, Abu-Elheiga LA.** Fatty acid metabolism: target for metabolic syndrome. *J Lipid Res* 50, *Suppl*: S138–S143, 2009. doi:10.1194/jlr.R800079-JLR200.
50. **Wheelock JB, Rhoads RP, Vanbaale MJ, Sanders SR, Baumgard LH.** Effects of heat stress on energetic metabolism in lactating Holstein cows. *J Dairy Sci* 93: 644–655, 2010. doi:10.3168/jds.2009-2295.
51. **Wilkins IA, Wheeler DW.** Regulation of temperature. *Basic Sci*: 168a-168e, 2004.
52. **Young AJ, Sawka MN, Levine L, Cadarette BS, Pandolf KB.** Skeletal muscle metabolism during exercise is influenced by heat acclimation. *J Appl Physiol (1985)* 59: 1929–1935, 1985. doi:10.1152/jappl.1985.59.6.1929.