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Abstract

Acute heat stress (HS) and heat stroke can be detrimental to the health, well-being, and performance of mammals such as swine. Therefore, our objective was to chronologically characterize how a growing pig perceives and initially copes with a severe heat load. Crossbred gilts (n=32; 63.8±2.9 kg) were subjected to HS conditions (37°C and 40% humidity) with ad libitum intake for 0, 2, 4, or 6 h (n=8/time point). Rectal temperature (Tr), respiration rates (RR), and feed intake were determined every 2 h. Pigs were euthanized at each time point and fresh ileum and colon samples were mounted into modified Ussing chambers to assess ex vivo intestinal integrity and function. Transepithelial electrical resistance (TER) and fluorescein isothiocyanate-labeled dextran (FD4) permeability were assessed. As expected, Tr increased linearly over time (P<0.001) with the highest temperature observed at 6 h of HS. Compared to the 0-h thermal-neutral (TN) pigs, RR increased (230%; P<0.001) in the first 2 h and remained elevated over the 6 h of HS (P<0.05). Feed intake was dramatically reduced due to HS and this corresponded with significant changes in plasma glucose, ghrelin, and glucose-dependent insulintropic peptide (P<0.050). At as early as 2 h of HS, ileum TER linearly decreased (P<0.01), while FD4 linearly increased with time (P<0.05). Colon TER and FD4 changed due to HS in quadratic responses over time (P=0.050) similar to the ileum but were less pronounced. In response to HS, ileum and colon heat shock protein (HSP) 70 mRNA and protein abundance increased linearly over time (P<0.050). Altogether, these data indicated that a short duration of HS (2-6 h) compromised feed intake and intestinal integrity in growing pigs.

Keywords

Animal Science, appetite, heat stress, intestinal integrity, swine

Disciplines

Animal Sciences | Food Biotechnology | Food Science | Human and Clinical Nutrition

Comments

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Short-term exposure to heat stress attenuates appetite and intestinal integrity in growing pigs¹

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ABSTRACT: Acute heat stress (HS) and heat stroke can be detrimental to the health, well-being, and performance of mammals such as swine. Therefore, our objective was to chronologically characterize how a growing pig perceives and initially copes with a severe heat load. Crossbred gilts ($n = 32$; 63.8 ± 2.9 kg) were subjected to HS conditions (37°C and 40% humidity) with ad libitum intake for 0, 2, 4, or 6 h ($n = 8$ /time point). Rectal temperature (T_r), respiration rates (RR), and feed intake were determined every 2 h. Pigs were euthanized at each time point and fresh ileum and colon samples were mounted into modified Ussing chambers to assess ex vivo intestinal integrity and function. Transepithelial electrical resistance (TER) and fluorescein isothiocyanate-labeled dextran (FD4) permeability were assessed. As expected, T_r increased linearly over time ($P < 0.001$) with the highest

temperature observed at 6 h of HS. Compared to the 0-h thermal-neutral (TN) pigs, RR increased (230%; $P < 0.001$) in the first 2 h and remained elevated over the 6 h of HS ($P < 0.05$). Feed intake was dramatically reduced due to HS and this corresponded with significant changes in plasma glucose, ghrelin, and glucose-dependent insulinotropic peptide ($P < 0.050$). At as early as 2 h of HS, ileum TER linearly decreased ($P < 0.01$), while FD4 linearly increased with time ($P < 0.05$). Colon TER and FD4 changed due to HS in quadratic responses over time ($P = 0.050$) similar to the ileum but were less pronounced. In response to HS, ileum and colon heat shock protein (HSP) 70 mRNA and protein abundance increased linearly over time ($P < 0.050$). Altogether, these data indicated that a short duration of HS (2–6 h) compromised feed intake and intestinal integrity in growing pigs.

Key words: appetite, heat stress, intestinal integrity, swine

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INTRODUCTION

Animal agriculture is severely affected by heat stress (HS) and the U.S. swine industry alone is estimated to lose over US\$300 million annually, while global losses are in the billions of dollars (St-Pierre et al., 2003). The fiscal losses are still observed despite recent advances in heat-abatement strategies. Growing pigs are highly susceptible to HS, which has also been shown to decrease performance (Song et al., 2011; Pearce et al., 2013a), nitrogen intake, and nitrogen retention (Brestensky

et al., 2012; Renaudeau et al., 2013). Additionally, HS pigs have altered metabolic responses (reduced heat production) and feeding behavior compared to thermoneutral-reared pigs (Renaudeau et al., 2013). We have also reported that HS reduced feed intake in pigs and the deleterious consequences of HS may also be partially mediated by its effects on intestinal integrity (Pearce et al., 2013a; Sanz Fernandez et al., 2014).

The gastrointestinal tract is highly sensitive to hyperthermic challenges as HS mammals redistribute blood to the periphery to maximize radiant heat dissipation. As a result, the intestine receives reduced blood and nutrient flow and this can compromise the intestinal barrier. This reduced intestinal integrity can increase lipopolysaccharide (LPS) in portal (Hall et al., 2001a) and systemic blood (Pearce et al., 2013b,c), which in turn can antagonize digestibility and anabolic pathways in pigs (Mani et al., 2012; Rakhshandeh et al., 2012).

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Interestingly, most studies addressed above have examined the long-term (24 h to 3 wk) impact HS has on a pigs' physiology and performance. However, few studies have examined how pigs initially perceive and adapt to HS within the first hours. Therefore, our objective was to chronologically characterize how growing pigs initially respond to and cope with a severe heat load. Specifically, we examined HS-induced chronological changes to appetite regulation and intestinal function.

MATERIALS AND METHODS

Animals and Study Design

All procedures were reviewed and approved by the Iowa State University Institutional Animal Care and Use Committee (number 2-12-7307-S). To assess how growing pigs perceive and cope with a high thermal load, 32 crossbred gilts (63.8 ± 2.9 kg BW) were used in a serial time course slaughter study. Gilts were randomly assigned to individual pens at the Iowa State University Swine Nutrition Farm (Ames, IA). All pigs were fed the same isoenergetic and isonitrogenous diet formulated to meet or exceed the predicted requirements (NRC, 1998) for energy, essential AA, protein, minerals, and vitamins (3,376 kcal/kg ME, 0.80% standardized ileal digestible lysine, and 4.6% crude fat). After a 4-d period to acclimate to diets and pens, each pig was assigned to 1 of 4 treatments based on BW. The treatments included 2 thermal environments and 3 durations: thermal-neutral (TN) conditions (21°C and approximately 70% humidity) for 6 h ($n = 8$) or HS conditions (37°C and approximately 40% humidity) for either 2 ($n = 8$), 4 ($n = 8$), or 6 h ($n = 8$). All pigs had free access to feed and water at all times. Each room's temperature and humidity were continuously monitored and recorded every 5 min by a data recorder (Lascar model EL-USB-2-LCD; Lascar Electronics, Erie, PA).

At the start of the experiment, all pigs were weighed and placed into their respective thermal climate conditions. Pigs were weighed, snared, and bled immediately before sacrifice at 2, 4, or 6 h of HS conditions or at 6 h of TN conditions (0-h HS). Pigs were sacrificed using a barbiturate overdose and exsanguination. During the experimental period, thermal status (rectal temperature [T_r]) and respiration rate [RR] and feed intake (FI) were recorded every 2 h and final TN cumulative FI was represented over the 6-h experimental period.

Blood was obtained via jugular venipuncture immediately before sacrifice. Blood was collected for plasma and serum using 10 mL K_2 EDTA, 10 mL serum clot, and 5 mL lithium heparin vacutainer tubes (BD, Franklin Lakes, NJ). Harvested blood was centrifuged at $1,300 \times g$ for 15 min at 4°C. Plasma and serum were obtained and

subsequently transferred into 1.5-mL microcentrifuge tubes and stored at -80°C for later analysis.

Intestinal tissues were harvested immediately following euthanasia and included whole sections from both the proximal ileum (approximately 2 m before the ileocecal junction) and distal colon (50 cm before the rectum). Fresh sections of whole ileum and colon were flushed of luminal contents, placed immediately into Krebs–Henseleit buffer (KHB; containing 25 mM NaHCO_3 , 120 mM NaCl, 1 mM MgSO_4 , 6.3 mM KCl, 2 mM CaCl, and 0.32 mM NaH_2PO_4 , pH 7.4) under constant aeration, and transported to the laboratory for mounting into Ussing chambers (Physiological Instruments, San Diego, CA). In addition, tissue samples were snap-frozen in liquid nitrogen and stored at -80°C until later analysis.

Blood Analyses

Blood from the lithium heparin vacutainers was immediately analyzed via an i-STAT handheld blood analyzer (EC8⁺ cartridge; Abaxis, Union City, CA). Analysis included measurement of sodium, potassium, chloride, TCO_2 , PCO_2 , HCO_3 , pH, glucose, blood urea nitrogen, hemoglobin, hematocrit, anion gap, and base excess of the extracellular fluid (BE_{ecf}).

Plasma insulin was analyzed in duplicate using an ELISA kit solid phase 2-site enzyme immunoassay based on the sandwich technique (Mercodia Porcine Insulin ELISA; ALPCO Diagnostics, Salem, NH) validated in our laboratory. The assay contained 5 calibrators as well as internal serum pools with low, intermediate, and high porcine insulin concentrations. The assay was conducted in 96-well microplates and read at 450 nm using a Synergy 4 microplate reader (Bio-Tek, Winooski, VT).

Plasma NEFA concentrations were determined using a commercially available kit (Wako HR Series NEFA-HR; Wako Diagnostics, Richmond, VA). Plasma urea nitrogen (PUN) was analyzed using a commercially available kit (Quantichrom Urea Assay Kit; BioAssay Systems, Hayward, CA).

Plasma samples were analyzed for lysozyme activity using a commercially available kit. Lysozyme activity was determined using the EnzChek fluorescent assay, which measures the lysozyme activity on *Micrococcus lysodeikticus* cell walls (Molecular Probes, Invitrogen, Carlsbad, CA). Fluorescence was measured in a Synergy 4 microplate reader (Bio-Tek) with excitation emission wavelengths of 485 and 530 nm. Activity was calculated using a standard curve constructed from assay standards. The assay detection range was 0 to 500 units/mL.

Alkaline phosphatase activity was measured using a commercially available kit. The QuantiChrom assay is a kinetic-based assay (BioAssay Systems). Briefly,

50 μL of sample was added to a 150- μL working solution containing magnesium, acetate, *p*-nitrophenyl phosphate, and assay buffer. Samples were read in a 96-well plate at 405 nm at time zero and again after 4 min on a Synergy 4 microplate reader (Bio-Tek). Furthermore, serum tumor necrosis factor- α (**TNF- α**) was also measured using a commercially available ELISA kit (Quantikine Porcine TNF- α , catalog number PTA00; R&D Systems, Minneapolis, MN). Serum haptoglobin samples were analyzed using a commercially available ELISA (ALPCO Diagnostics).

Plasma LPS-binding protein (**LBP**) concentrations were determined using a commercially available ELISA (LBP human ELISA kit; Hycult Biotech, Plymouth Meeting, PA). Serum endotoxin concentrations were determined using a commercially available kit validated for use in our laboratory. Endotoxin concentrations were determined in triplicate using a recombinant Factor C endotoxin assay with a 1:1,000 dilution factor for porcine plasma samples (PyroGene Recombinant Factor C Endotoxin Detection System; Lonza, Walkersville, MD). The procedure was conducted in 96-well microplates, and fluorescence was measured at time 0 and after 1 h of incubation at 37°C. The plates were then read under fluorescence using a Synergy 4 microplate reader (Bio-Tek) with excitation/emission wavelengths of 380/440 nm. Relative fluorescence unit (**RFU**) was determined, and concentration of endotoxin was interpolated from the standard curve. Endotoxin concentrations (RFU) were expressed as arbitrary units.

To determine the plasma concentration of hormones related to appetite and glycemia, 4 mL of blood was drawn into EDTA-coated vacutainer tubes (BD, Franklin Lakes, NJ.) and immediately mixed with 400 μL of 10,000 KIU (1.4 mg/mL) aprotinin and centrifuged at 3,000 $\times g$ at 4°C for 15 min. The plasma was then divided into aliquots and stored at -80°C until analysis. Gastric inhibitory peptide, also known as glucose-dependent insulinotropic peptide (**GIP**), was by measured Radioimmunoassay (**RIA**) using 1:5,000 rabbit anti-human GIP antibody (Phoenix Pharmaceuticals, Burlingame, CA); the assay had a detection range of 0.1 to 6.4 ng/mL, with an intra-assay CV of 7% and an interassay CV of 12% at 0.5 ng/mL. Plasma cholecystokinin (**CCK**) was assayed by RIA using rabbit anti-CCK-8 antibody 92128 diluted in the ratio 1:800 (Phoenix Pharmaceuticals). The assay was able to measure plasma CCK concentration from 4 to 128 pg/mL. The intra-assay CV was 8% and the interassay CV was 15% at 50 pg/mL. Ghrelin was analyzed by RIA using antibody T-4745 purchased from Bachem (Torrance, CA). The assay had a determination range of 0.05 to 12.8 ng/mL. The intra-assay CV was 10% and the interassay CV was 7% at 0.5 ng/mL. 125I-Tracers used for RIA were purchased from PerkinElmer (Waltham, MA).

Ex Vivo Intestinal Integrity Measures

Ileal and colonic segments from each animal were mounted into modified Ussing chambers (Physiological Instruments and DVC 1000; World Precision Instruments, New Haven, CT) for determination of intestinal integrity and macromolecule transport. Tissue samples were pinned and placed vertically into the chambers with the mucosal membrane facing one-half of the chamber and the serosal membrane facing the other half. Each side of the membrane was bathed in 4 mL of KHB and tissue was provided with a constant O₂-CO₂ mixture. Individual segments were then voltage clamped (0 mV), and after 20 min of stabilization, transepithelial electrical resistance (**TER**) was calculated by averaging the current during the first 10 min of tissue stabilization (Gabler et al., 2007).

Ileum and colonic segments were also assessed for 4.4 kDa macromolecule transport using fluorescein isothiocyanate-labeled dextran (**FD4**; Sigma, St. Louis, MO). After 20 min of stabilization, KHB was replaced with fresh buffer on both the mucosal and the serosal sides of the chamber. In addition, the mucosal side received 2.2 mg/mL of FD4. Samples from the serosal side were obtained in duplicate every 20 min for 120 min and read in a fluorescence spectrophotometer (495 nm excitation), and an apparent permeability coefficient was calculated as previously described by Mani et al. (2012).

Protein Expression

Whole cell protein from ileum and colon was extracted and separated by SDS-PAGE, and semiquantitative protein abundance of heat shock protein (**HSP**) 70 and hypoxia inducible factor-1 α (**HIF-1 α**) were determined as previously describe (Pearce et al., 2013b,c). Ileum mucin 2 (**MUC2**) protein abundance was determined using a commercially available ELISA (Porcine MUC 2 ELISA kit; MyBioSource, San Diego, CA) after whole ileum proteins were extracted using PBS (pH 7.2) containing protease inhibitors. The sample protein concentration was determined using bicinchoninic acid assay (Pierce, Rockford, IL), and MUC2 was expressed as nanograms of mucin 2 MUC2 per milligram of protein.

Messenger RNA Abundance Analysis

Total RNA was isolated from ileum tissue using a commercially available kit (RNeasy fibrous tissue mini kit; Qiagen, Valencia, CA). Total RNA was quantified and cDNA was synthesized for real-time quantitative PCR as previously described (Pearce et al., 2013c). Real-time quantitative polymerase chain reaction (**qPCR**) was performed using a BioMark HD system (Fluidigm Corporation, San Francisco, CA). Complementary

Table 1. Time course effects of heat stress on appetite peptides and blood metabolites¹

Parameter	Time, h				SE	P-value		
	0	2	4	6		Time	Linear	Quadratic
Plasma GIP, ² pg/mL	287.3 ^a	126.5 ^b	53.3 ^c	50.0 ^c	29.30	<0.001	<0.001	0.891
Plasma ghrelin, pg/mL	1,618 ^a	2,033 ^{ab}	1,861 ^a	2,331 ^b	174.0	0.049	0.018	0.126
Plasma CCK, ³ pg/mL	4.93	3.74	3.13	2.44	0.836	0.170	0.030	0.858
Plasma insulin, ng/dL	0.14	0.12	0.08	0.09	0.0254	0.327	0.091	0.572
Plasma NEFA, mmol/L	0.13	0.18	0.15	0.19	0.023	0.174	0.139	0.083
Plasma glucose, mg/dL	93.7 ^a	112.4 ^b	111.6 ^b	100.4 ^{ab}	5.12	0.035	0.402	0.681
Plasma urea nitrogen, mg/dL	60.7	58.7	67.9	57.2	7.79	0.766	0.972	0.378

^{a-d}Means within a row with different superscripts differ ($P < 0.05$).

¹0, 2, 4, or 6 h of heat stress (37°C and approximately 40% humidity; $n = 8$ /time point).

²GIP = gastric inhibitory peptide.

³CCK = cholecystokinin.

DNA from tissues was used for specific target amplification using the TaqMan PreAmp Master Mix (Life Technologies, Grand Island, NY) and loaded onto Fluidigm's Dynamic Array Integrated Fluidic Circuits (IFC) according to Fluidigm's EvaGreen DNA Binding Dye protocols. Gene symbols, accession numbers, and primer sequences are listed in Supplemental Table 1. One 48.48 Dynamic Array IFC plate was used to analyze mRNA abundance of selected genes in porcine ileum and colon mucosal scraping tissues. Four genes (RPL32, ACTB, TOP2B, and GAPDH) were included into the qPCR array to select for endogenous reference genes. The mRNA abundance values for each sample were normalized to RPL32; its abundance was not altered ($P > 0.050$) by treatment, according to the $2^{\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Intestinal Histology

Whole ileum samples fixed in formalin were sent to the Iowa State University Veterinary Diagnostic Laboratory (Ames, IA) for sectioning and hematoxylin and eosin staining. Using a microscope (DMI3000 B Inverted Microscope; Leica Microsystems, Bannockburn, IL) with an attached camera (12-bit QICAM Fast 1394; QImaging, Surrey, BC, Canada), images were taken of 10 oriented villi and crypts per section on 3 sections. Then, within pig, the average of the 30 villus height and crypt depth measurements was calculated and used for statistical analyses. Images of individual villi and crypts were obtained using Q-capture Pro 6.0 (QImaging) and measurements were taken using Image-Pro Plus 7.0 (Media Cybernetics, Bethesda, MD).

Statistical Analysis

All data were statistically analyzed using the PROC MIXED procedure of SAS version 9.2 (SAS Inst. Inc., Cary NC). Data are reported as least square means and

considered significant if $P \leq 0.050$ and a tendency if $P \leq 0.100$. The model included comparison time (0, 2, 4, and 6 h of HS) as a fixed effect. Additionally, linear and quadratic contrast statements were used to further examine parameter changes over time. For hourly measurements (T_r and RR), each animal's respective parameter was analyzed using repeated measures with an autoregressive covariance structure and time as the repeated effect. The repeated measures model used fixed effects of treatment, time, and treatment \times time.

RESULTS

Phenotypic Measures

Pig T_r increased from 39.2 to 41.2°C from 0 to 2 h of HS (2°C increase) and remained elevated throughout the 6-h period ($P < 0.001$; Fig. 1a). However, there was no change between 2, 4, and 6 h of HS ($P > 0.050$). Respiration rates more than doubled from 0 to 2 h of HS (50.5 vs. 119.5 breaths per min; $P < 0.001$; Fig. 1b). However, again there was no difference between 2, 4, and 6 h of HS ($P > 0.050$). Feed intake was measured every 2 h for 6 h and the 0-h HS pigs ate 1.1 kg in 6 h (Fig. 1c). As expected, FI was dramatically reduced due to HS at 2, 4, and 6 h compared to the TN pigs ($P < 0.050$; Fig. 1c). However, FI did not differ between 2, 4, and 6 h of HS ($P > 0.050$). An approximately 80% reduction in FI was observed between the TN group at 6 h and the 6-h HS group ($P < 0.050$). The TN pigs gained 1.01 kg BW over the 6-h period, while HS pigs at each time point lost significantly more weight in a linear manner (-1.03, -2.38, and -3.00 kg, respectively; $P < 0.001$).

Appetite Regulation and Blood Metabolites

There was a linear decrease in plasma CCK concentration as HS duration increased ($P = 0.030$; Table 1). Plasma GIP also was significantly decreased

over time in a linear manner due to HS ($P < 0.001$; Table 1). Compared to the TN pigs, a 55.9% reduction in GIP concentration was recorded after 2 h of HS ($P < 0.001$) and this was reduced by 81% after 4 and 6 h of HS ($P < 0.001$; Table 1). Plasma ghrelin concentrations increased in a linear manner as HS duration increased ($P = 0.049$). Compared to the TN control pigs, ghrelin concentrations increased 140% by 6 h of HS ($P < 0.050$). Plasma insulin ($P = 0.327$), NEFA ($P = 0.174$), and PUN ($P = 0.766$) concentrations did not differ across time (Table 1). However, there was a significant HS time effect on plasma glucose concentrations ($P = 0.035$; Table 1), which increased 120% by 2 and 4 h of HS compared to the TN control pigs.

Protein Expression

To assess the stress response to HS, ileum HSP70 and HIF-1 α protein expression was measured (Fig. 2). There were no significant changes in ileum HSP70 ($P = 0.564$) and HIF-1 α ($P = 0.131$) protein expression between the TN and HS pigs (Fig. 2). However, there was a significant linear increase ($P = 0.023$; Fig. 2) in ileum HSP70 expression but not HIF-1 α expression ($P = 0.112$) over time. Colon HSP70 protein expression was significantly increased starting at 2 h of HS and remained significantly elevated until 6 h compared to the TN controls ($P < 0.050$; Fig. 2). Colon HIF-1 α was not different due to HS duration; however, there was a tendency ($P = 0.064$) for a linear increase in expression over time.

Ex Vivo Intestinal Integrity and Histology Measures

Ileum TER was decreased due to HS, irrespective of duration (38%; $P = 0.005$). These findings were mirrored by an increase in ileal FD4 permeability in HS pigs compared to TN controls (34.8 vs. 2.9 cm/min, respectively; $P = 0.032$; Table 2). Again, no differences in FD4 permeability were observed between 2-, 4-, and 6-h HS durations. Colon TER ($P = 0.595$) and FD4 ($P = 0.409$) were not altered due to HS in this short period of time (Table 2).

Ileum villi length was reduced due to HS compared to the TN samples ($P < 0.001$; Table 2; Fig. 3), and this reduction in height was more severe as HS duration increased ($P < 0.001$). Compared to the TN controls, the largest decrease occurred at 6 h of HS (37.6% lower length). However, ileum crypt depth tended to increase with HS duration compared to the TN crypt depth ($P = 0.091$; Table 2; Fig. 3). The ratio of villi height to crypt depth was significantly decreased by HS ($P < 0.001$; Table 2; Fig. 3), again with the largest decrease occurring at the 6-h time point (34.7% lower; $P < 0.001$). Increased villi tip autolysis was also observed in the

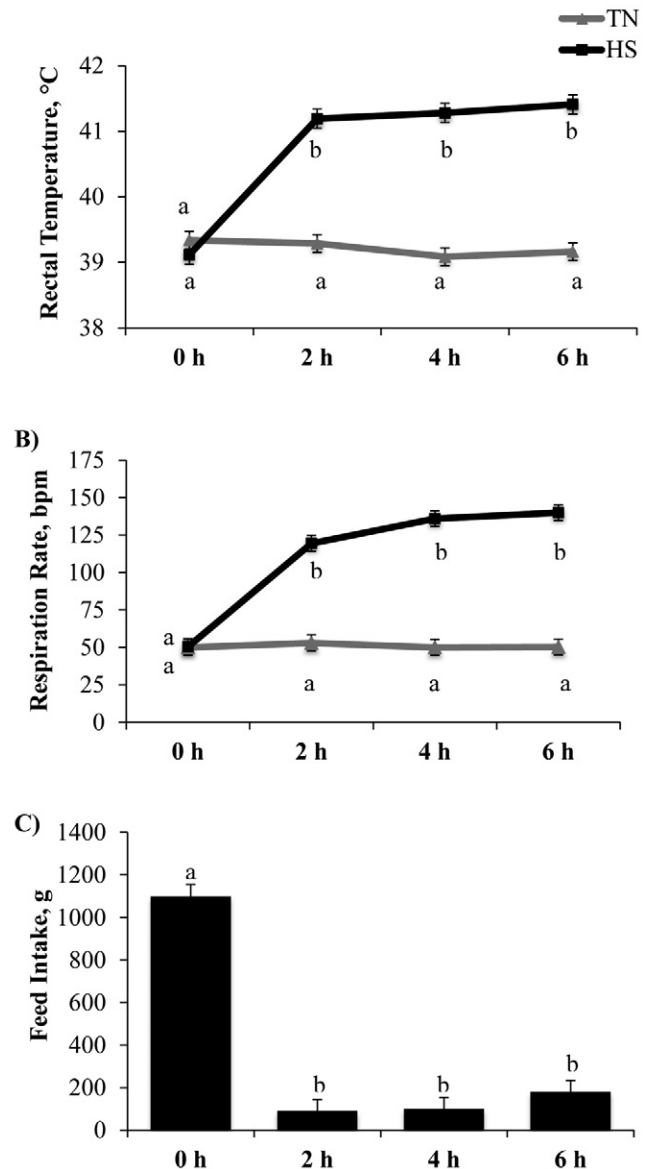


Figure 1. Chronological effects (0–6 h) of heat stress (HS) exposure (37°C and approximately 40% humidity) on A) rectal temperature, B) respiration rates, and C) feed intake. Zero-hour heat stress treatment represents 6 h of thermal-neutral (TN; 21°C and approximately 70% humidity) data collection. $n = 8$ /time point. ^{a,b} $P < 0.001$. bpm = breaths.

histology sections of 4- and 6-h HS pigs (Fig. 3). To further assess changes in ileum integrity, MUC2 protein expression was examined and shown to be increased (67%) at only 6 h compared to the 0-h TN and 2- and 4-h HS groups ($P < 0.001$; Table 2).

Blood Inflammatory Markers

Plasma LBP was decreased 29.3% ($P < 0.050$; Fig. 4) in 2-h HS pigs compared to TN pigs. Plasma LBP was also decreased at 4 and 6 h (26.4 and 43.9%, respectively; Fig. 4) but it was not significantly altered between the 3 HS time points ($P > 0.050$). Serum endotoxin was not significantly altered due to HS ($P >$

Table 2. Time course effects of heat stress on intestinal integrity and morphology¹

Parameter	Time, h				SEM	P-value		
	0	2	4	6		Time	Linear	Quadratic
Ileum TER, ² $\Omega \cdot \text{cm}^2$	185 ^a	118 ^b	112 ^b	116 ^b	20.4	0.005	0.008	0.559
Colon TER, $\Omega \cdot \text{cm}^2$	105	91	93	102	10.3	0.595	0.838	0.870
Ileum FD4, ³ permeability	2.9 ^a	28.8 ^b	38.0 ^b	37.7 ^b	11.27	0.032	0.015	0.882
Colon FD4, permeability	8.8	20.8	24.4	27.5	10.84	0.409	0.156	0.865
Ileum mucin 2, ng/g protein	0.52 ^a	0.49 ^a	0.48 ^a	0.87 ^b	0.060	<0.001	<0.001	0.021
Ileum morphology								
Villi height, μm	491 ^a	448 ^b	373 ^c	310 ^d	19.6	<0.001	<0.001	0.675
Crypt depth, μm	257	302	285	245	16.5	0.091	0.491	0.576
Villi: crypt ratio	1.96 ^a	1.52 ^b	1.32 ^b	1.28 ^b	0.095	<0.001	<0.001	0.852

^{a-d}Means within a row with different superscripts differ ($P < 0.05$).

¹0, 2, 4, or 6 h of heat stress (37°C and approximately 40% humidity; $n = 8/\text{time point}$).

²TER = transepithelial electrical resistance.

³FD4 = fluorescein isothiocyanate-labeled dextran (4.4 kDa permeability, cm/min).

0.05; Fig. 4). However, there was a tendency ($P = 0.100$) for a linear increase in serum endotoxin over time. There were no significant differences in plasma lysozyme, serum haptoglobin, or serum alkaline phosphatase concentrations due to the duration of HS ($P > 0.050$;

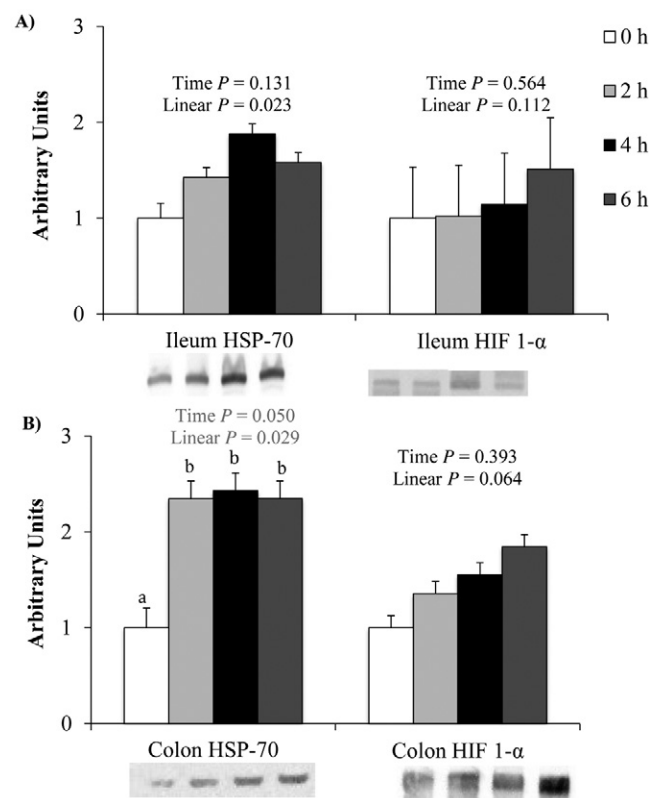


Figure 2. Temporal effects of 0, 2, 4, or 6 h of heat stress exposure (37°C and approximately 40% humidity) on A) ileum and B) colon heat shock protein (HSP) 70 and hypoxia inducible factor-1 α (HIF-1 α) protein expression. Zero-hour heat stress treatment represents 6 h of thermal-neutral (21°C and approximately 70% humidity) data collection. $n = 8/\text{time point}$ and all semiquantitative data was adjusted relative to glyceraldehyde 3 phosphate dehydrogenase protein expression. ^{a,b} $P \leq 0.050$.

Table 3). However, serum TNF- α concentration differed significantly over time ($P = 0.002$; Table 3).

Compared to the TN control pigs, plasma sodium was increased due to HS, irrespective of time ($P = 0.040$; Table 4) and in a linear manner ($P = 0.013$). Potassium was decreased due to HS, irrespective of time and in linear fashion ($P = 0.037$; Table 3). Chloride was increased due to HS, again irrespective of time point ($P = 0.020$; Table 3). Blood pH was increased from 7.39 at 0 h of HS to 7.49 at 2 h of HS ($P = 0.013$; Table 3) and remained elevated at 4 and 6 h of HS. Partial CO₂ was linearly decreased due to HS duration ($P < 0.001$; Table 3). There were no differences in hematocrit, bicarbonate, anion gap, hemoglobin, or BEecf due to HS ($P > 0.050$; Table 3).

Ileal mRNA Abundance

Changes in ileum tissue mRNA abundance due to 2 to 6 h of HS is reported in Table 4. As expected, HS induced a rapid transient increase in *HSP27*, *HSP70*, and *HSP90AA1* ($P < 0.050$). However, the transcription factor HSF1 mRNA was not different across treatments ($P = 0.499$). Hypoxia inducible factor-1 α tended ($P = 0.096$) to decrease by 6 h while there were no changes to hypoxia inducible factor-2. There were no time, linear, or quadratic differences with *Heme oxygenase* or *Acyloxyacyl hydrolase* ($P > 0.050$). There was a quadratic trend ($P = 0.072$) for *Na⁺/K⁺ ATPase* to be highest at 4 h and then decrease at 6 h. There was also a linear trend ($P = 0.079$) for *CLDN3* to be increased over time. There were no differences in *occludin* (*OCLN*) or *Myosin light chain kinase* ($P > 0.050$). For *MCT7*, there was no difference in gene abundance ($P = 0.733$). Mucin 2 gene expression had a linear trend ($P = 0.072$) as it increased at 2 h and quickly fell below baseline levels

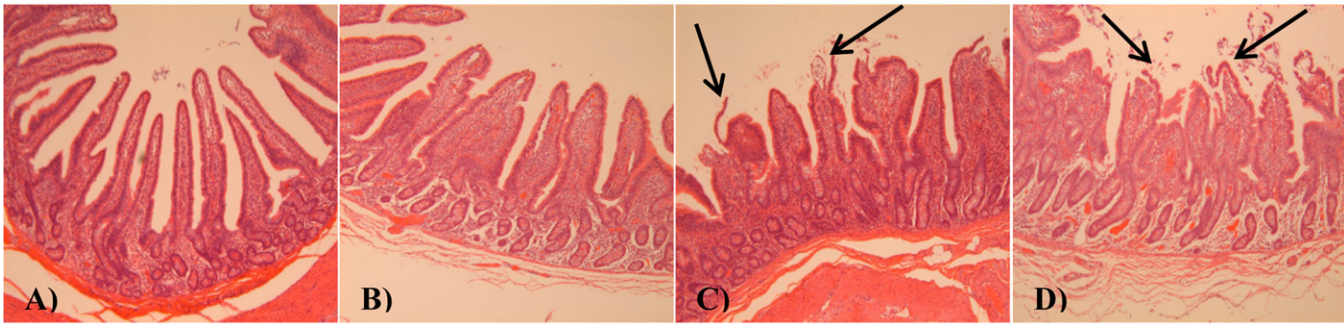


Figure 3. Temporal changes in the ileum morphology due to heat stress (HS; 37°C and approximately 40% humidity). A) 0 h of HS exposure, B) 2 h of HS, C) 4 h of HS and D) 6 h of HS exposure. Note the shortening of the villi length and increased autolysis of the villi tips as the duration of heat stress exposure escalates. The black arrows indicate areas of autolysis. Zero-hour HS treatment represents thermal-neutral (21°C and approximately 70% humidity) conditions. See online version for figure in color.

by 4 and 6 h. There was a tendency for a quadratic trend for mucin 4 (MUC4) to be increased by 2 h and then decreased by 4 and 6 h ($P = 0.062$). Ubiquitin (*UBB*) gene expression was increased ($P < 0.050$) in HS pigs by 2 and 4 h; however, it returned to baseline levels by 6 h. There were no changes in *IAP*, *ITGB1*, *TGFB1*, *IL1B*, *IL6*, *SLC5A1*, or *SLCA2* gene expression ($P > 0.050$).

DISCUSSION

Although HS clearly has been shown to alter pig performance and health indices, there is limited data characterizing how pigs first perceive and cope with a high ambient heat load. Characterizing the early hours of HS or heat stroke is critical for determining future prevention and/or mitigation strategies. Therefore, we examined chronological changes in appetite and intestinal function and integrity in growing pigs over a 6-h HS period (constant 37°C and 40% humidity). These conditions were chosen to mimic the physiological response to a short but severe HS load that was well above the pig's upper critical temperature range. Thus, by design, pigs experienced a heat load well above their thermal comfort zone, and this resulted in a marked increase in body thermal indices. The average difference in T_r between TN pigs and HS pigs was 2.1°C, which is consistent with what has been previously reported (Johnson et al., 2013).

As expected, exposure to HS caused a sharp decrease in FI and BW. This is a significant amount of weight loss in such a short time, and we think that much of this is likely due to reduced gut fill but also may be due to changes in basal metabolic rate, increased motility, and water loss. The percentage of FI decrease and BW loss are much higher than observed in other studies (Collin et al., 2001; Renaudeau et al., 2008), but we believe this is due to the severity of our heat load and may be related to appetite regulation.

Reduced FI is a highly conserved response among species under HS. It is thought that animals reduce FI to reduce metabolic heat production as this has been

shown in previous research (Renaudeau et al., 2013) and that neuropeptide secretions from the hypothalamus and intestines may also be involved. Previous studies in heat-stressed broilers found that mRNA levels of ghrelin increased in the stomach and intestines after acute HS, together with a decrease in CCK mRNA abundance in the duodenum and hypothalamus (Song et al., 2012; Lei et al., 2013). The reduction in FI due to HS may be mediated mainly by activation of peripheral ghrelin secretion (Lei et al., 2013).

In agreement with the previous work done in poultry, we report a decrease in GIP herein. This hormone reduces gastric motility and blocks gastric secretion as well as induces insulin secretion and stimulates lipoprotein lipase activity (Ugleholdt, 2011). A decrease in GIP would mean increased gastric motility and gastric secretion. We also observed an increase in circulating ghrelin concentration. Ghrelin is secreted when the stomach is empty and increases hunger signals as well as gastric acid secretion and increases gastrointestinal motility (Sobrinho et al., 2014). Our ghrelin data also points to an increase in gastric motility and acid secretion as ghrelin

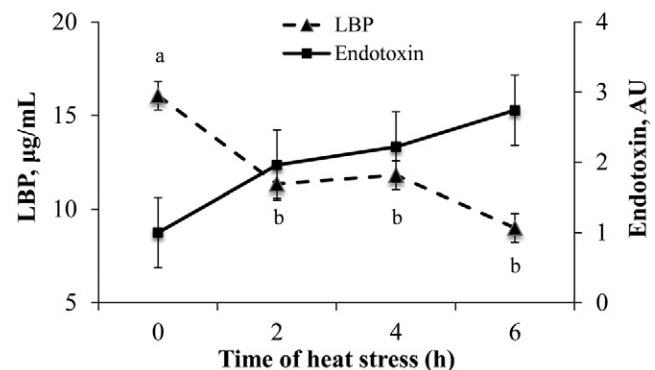


Figure 4. Effects of 6 h thermal-neutral conditions (21°C) or 2, 4, or 6 h of heat-stress conditions (37°C and approximately 40% humidity) on serum endotoxin and plasma lipopolysaccharide-binding protein (LBP) concentrations. Zero-hour heat stress treatment represents 6 h of thermal-neutral (21°C and approximately 70% humidity) data collection $n = 8$ /time point. $a, bP = 0.039$. AU = arbitrary unit.

Table 3. Chronological effects of heat stress on blood inflammatory, immune, and gas markers

Parameter	Time, h ¹				SEM	P-value		
	0	2	4	6		Time	Linear	Quadratic
Inflammation markers								
Tumor necrosis factor- α , pg/mL	68.4 ^a	57.5 ^b	68.3 ^a	48.7 ^b	3.31	0.002	0.006	0.004
Haptoglobin, mg/mL	72.5	64.3	79.1	79.4	15.00	0.879	0.605	0.581
Lysozyme, units/mL	98.9	73.7	88.2	82.6	9.57	0.299	0.424	0.151
Alkaline phosphatase, IU/L	50.2	52.1	47.6	48.4	5.96	0.953	0.712	0.671
Ion and gas markers								
Sodium, mmol/L	135.0 ^a	140.9 ^b	143.6 ^b	142.8 ^b	2.18	0.040	0.013	0.960
Potassium, mmol/L	6.20 ^a	4.90 ^b	4.45 ^b	5.03 ^b	0.414	0.037	0.041	0.925
Chloride, mmol/L	99.3 ^a	102.5 ^b	104.3 ^b	104.4 ^b	1.20	0.020	0.004	0.967
Hematocrit, % PVC ²	38.9	38.1	37.1	36.9	0.92	0.380	0.091	0.794
pH	7.39 ^a	7.49 ^b	7.47 ^b	7.46 ^b	0.021	0.013	0.054	0.222
Partial CO ₂ , kPa	6.5 ^a	5.1 ^b	4.9 ^b	5.0 ^b	0.27	<0.001	<0.001	0.421
Bicarbonate, mmol/L	28.9	27.8	25.5	26.5	1.21	0.193	0.074	0.374
Anion gap, mmol/L	15.83	15.63	18.14	29.13	7.611	0.451	0.193	0.853
Hemoglobin, g/dL	13.31	12.95	12.64	12.54	0.289	0.247	0.051	0.901
BE _{ecf} ³ , mmol/L	3.13	4.13	1.50	2.75	1.407	0.624	0.556	0.243

^{a-d}Means within a row with different superscripts differ ($P < 0.05$).

¹0 h (thermal neutral) or 2, 4, or 6 h of heat stress (37°C and approximately 40% humidity; $n = 8$ /time point).

²PVC = Packed cell volume.

³BE_{ecf} = base excess of the extracellular fluid.

is secreted when the stomach is empty and increases gastric acid secretion and gastrointestinal motility. Finally, CCK was also decreased linearly due to HS in the current study. This hormone is normally responsible for inhibiting gastric emptying and gastric acid secretion. It also stimulates digestive enzyme secretion from the pancreas (Dockray, 2012). This brain–gut signaling may be a conserved way to signal to the animal that eating during HS is detrimental as it increases metabolic heat production. Although our sampling procedures were not tightly controlled (animals were fed ad libitum and there was only 1 sampling time point per animal) for measuring neuropeptides, we believe this still provides new insight on appetite signaling during HS in the pig.

The deleterious effects of HS are mediated, at least in part, by its effects on gastrointestinal health and function. During HS, blood flow is diverted from the splanchnic system to the skin in an attempt to dissipate excess heat (Lambert, 2009). Reduced blood flow and hyperthermia leads to hypoxia and oxidative and nitrosative stress in the enterocyte (Lambert, 2004; Pearce et al., 2013c). As a result, cell membranes and tight junctions can be damaged, leading to an increase in intestinal permeability, and this is also known as “leaky gut” (Lambert et al., 2002). This was clearly evident in our 6-h HS histology images in which HS increased autolysis of the ileum epithelium. Heat stress–induced damage to the intestines has been shown to increase the passage of high molecular weight substances and pathogens, including LPS and other bacterial components from the lumen (Pearce et al., 2013b). Interestingly, we have now shown these changes

in intestinal integrity are occurring as early as 2 h after HS, particularly in the small intestine.

In rodents (Lambert et al., 2002) and poultry (Song et al., 2013, 2014), HS significantly affects small intestinal permeability as assessed by either TER or FD4 permeability. Even within a short time period (60 minutes), heat-stressed rodents at 41.5 to 42°C core temperature had increased FD4 permeability. This was consistent across all areas of the intestinal tract, with the lowest permeability seen in the colon (Lambert et al., 2002). This similarly fits with our data as we see greater permeability at 6 h in the ileum compared to colon. The magnitude of the TER change we observed was somewhat similar to a previous study in large size pigs (Albin et al., 2007). Histologically, as little as 30 min of severe HS (41.5–42°C) has been shown to cause sloughing of villi tips and, by 60 min, entire epithelial sheets sloughing as well as denuded villi in rodents (Lambert et al., 2002). Electron micrographs at the same time points also showed massive intracellular swelling and damage to microvilli by 60 min of HS (Lambert et al., 2002). Our data suggests that in pigs exposed to HS, at as early as 2 h we observed villi shortening and by 4 to 6 h we see epithelial sloughing, autolysis, and linear lamina separation. This fits similarly with heat-stressed mice, which had lesions in the small intestine (Leon et al., 2006), and also with those reported in heat stroke cases in which endotoxemia was observed (Hall et al., 2001a; Lambert et al., 2002).

Regionally, heat stroke in mice appears to affect the upper small intestine (duodenum and jejunum) more

Table 4. Temporal effects of heat stress on ileum mRNA abundance

Parameter	Time, h ¹				SEM	P-value		
	0	2	4	6		Time	Linear	Quadratic
Heat shock protein 27	0.32 ^a	0.89 ^{ab}	1.64 ^b	1.17 ^b	0.358	0.015	0.011	0.237
Heat shock protein 70	0.06 ^a	2.51 ^b	1.03 ^b	0.11 ^a	0.742	0.015	0.574	0.076
Heat shock protein 90	0.20 ^a	1.13 ^{bc}	2.00 ^c	0.92 ^b	0.368	0.002	0.020	0.123
Heat shock factor 1	0.66	0.61	0.82	0.66	0.139	0.499	0.666	0.177
Hypoxia inducible factor 1 α	0.88	0.90	0.92	0.68	0.099	0.096	0.084	0.397
Hypoxia inducible factor 2	0.53	0.68	0.98	0.72	0.258	0.388	0.306	0.396
Heme oxygenase	0.22	0.54	0.43	0.38	0.111	0.231	0.450	0.293
Acyloxyacyl hydrolase	0.81	0.90	1.13	0.77	0.205	0.616	0.920	0.442
Claudin-3	0.58	0.59	1.13	1.00	0.216	0.202	0.079	0.230
Claudin-4	0.69	0.81	1.15	0.65	0.254	0.515	0.846	0.368
Occludin	0.93	0.66	1.17	0.74	0.226	0.412	0.944	0.107
Myosin light chain kinase	0.83	0.94	1.60	0.92	0.523	0.710	0.696	0.426
Mucin 2	0.41	0.66	0.86	0.80	0.223	0.231	0.072	0.760
Mucin 4	0.67	0.94	0.30	0.31	0.245	0.079	0.062	0.061
Trefoil factor-2	0.88	0.67	0.84	0.67	0.282	0.925	0.714	0.581
Mast cell tryptase	0.93	0.69	0.78	0.56	0.231	0.733	0.351	0.550
Intestinal alkaline phosphatase	0.90	0.84	0.98	0.84	0.357	0.978	0.979	0.683
Integrin β -1	0.75	0.96	1.50	0.98	0.203	0.098	0.198	0.138
Transforming growth factor β -1	0.65	0.51	0.59	0.54	0.097	0.748	0.557	0.430
Interleukin 1- β	0.78	0.52	0.98	0.51	0.215	0.379	0.724	0.107
Interleukin 6	4.57	4.42	3.85	2.50	1.974	0.869	0.454	0.966
Na ⁺ /K ⁺ ATPase	0.70	0.69	1.23	0.80	0.223	0.178	0.314	0.072
Sodium-glucose transporter 1	0.63	0.81	1.41	0.76	0.273	0.230	0.436	0.192
Glucose transporter 2	0.89	9.88	1.27	0.65	0.257	0.412	0.759	0.239

^{a-c}Means within a row with different superscripts differ ($P < 0.05$).

¹0, 2, 4, or 6 h of heat stress (37°C and approximately 40% humidity; $n = 8$ /time point).

severely than the ileum, and this matches up with higher permeability of FD4 in the proximal vs. distal areas of the small intestine (Novosad et al., 2013). Systemically, changes in intestinal integrity were accompanied by an increase in circulating endotoxin. Increased circulating endotoxin during HS has been reported in several previous studies (Hall et al., 2001a; Lambert, 2008; Wang et al., 2011; Pearce et al., 2013b) and our current results fit with previous data. Interestingly, we also observed a decrease in circulating LBP. Lipopolysaccharide-binding protein is an acute phase protein that interacts with and binds LPS molecules, subsequently presenting them to CD14 to initiate an immune response (Lu et al., 2008). We hypothesize that a free form of circulating LBP is decreased over time as endotoxin increases and is being bound up by LBP molecules.

Similar to our previous work (Pearce et al., 2013c), we reported no changes in small intestinal mRNA abundance of tight junction proteins (except for an increase in claudin-3 expression) due to HS, even though we report an attenuated integrity phenotype. However, in vitro data has shown that protein expression of tight junction proteins was negatively affected in a time-dependent manner due to a 41°C heat exposure (Dokladny et al., 2006). As early as 8 to 12 h, these

authors reported occludin protein expression to be increased in Caco-2 cells while ZO-1 is decreased. Additionally, occludin expression in Caco-2 cells exposed to 41°C was increased as early as 4 h while ZO-1 started to decrease at 6 h (Dokladny et al., 2008). These data suggest that changes in mRNA abundance of tight junction proteins due to HS may be more related to the protein phenotype than transcriptional. Interestingly, although TER decreased as early as 2 h in the current study, we did not observe an increase in ileal MUC2 until 6 h and this was corroborated by an increase in *MUC2* gene expression. Mucin 2 is a main secretory form of mucin, which is secreted from intestinal goblet cells and acts as a protective barrier for the intestine. During ischemia, mucin addition to intestinal epithelial cells has been shown to improve intestinal permeability in rodent small intestine (Chang et al., 2012), and in heat-stressed chickens, mucin producing goblet cells are increased in the ileum (Ashraf et al., 2013). Our increase in mucin expression may be a compensative mechanism to try and combat the decrease in intestinal integrity. Another integrity-related gene, which was increased due to HS, was integrin β -1 ITGB1. Integrins are a family of transmembrane proteins involved in cell structure and are expressed in intestinal mucosa. A previous study

using a necrotizing enterocolitis model showed that enterocyte migration is inhibited by LPS via increased expression of integrin proteins, thus impairing intestinal restitution after injury (Qureshi et al., 2005).

A classical response to HS can be observed through the heat shock response orchestrated through HSP. Heat shock proteins are molecular chaperones, which aid in protein folding and cell survival under stress. In the present study, we observed a transient increase in the mRNA and protein expression of several HSP including *HSP27*, *HSP70*, and *HSP90* and an increase in protein expression in the ileum and colon of *HSP70*. Dokladny et al. (2006) has shown *HSP70* expression upregulated within 2 h of heat exposure in Caco-2 cells, with the maximal levels by 4 to 8 h. A similar pattern was shown in the same study with *HSP27*, *HSP40*, and *HSP90*. Heat shock proteins prefer to localize in the more severe microenvironments such as the acidic stomach or the highly bacterial colon (Tanguay et al., 1993). This may partially explain why we have higher expression in the colon compared to the ileum. Also, differences in HSP accumulation between varying tissues may be related to actual tissue temperature differences, as the temperature of internal organs during HS may be lower than peripheral tissues such as muscle (Flanagan et al., 1995). Interestingly, in terms of *HSP72* specifically, liver, small intestine, and kidney were affected before other peripheral tissues such as brain and muscles (Flanagan et al., 1995), supporting the notion that the intestines are one of the first and more susceptible organs negatively impacted by a severe heat load.

In the current study, we also observed changes to HIF protein and gene expressions. Under hypoxic conditions, HIF-1 α is rapidly upregulated to support many aspects of cell survival (Eltzschig and Carmeliet, 2011). Protein expression of HIF-1 α increased in both the ileum and colon of HS pigs while gene expression of *HIF-1 α* decreased. Although the gene expression data did not match our protein data, we have reported a similar upregulation of this protein in a previous HS study (Pearce et al., 2013b).

Heat stress markedly alters protein metabolism in a number of animal species independent of FI reduction (Kamiya et al., 2006; Wheelock et al., 2010). In this study, HS increased PUN and this agrees with previous studies in cows (O'Brien et al., 2010; Wheelock et al., 2010). Urea is synthesized in the liver from ammonia generated from both exogenous (i.e., dietary) and endogenous (i.e., skeletal muscle) protein catabolism. Additionally, previous HS studies have shown an increase in 3-methylhistidine, a better marker of muscle catabolism (Yoshizawa et al., 1997; Pearce et al., 2013a). In the current study, we did not observe changes in PUN over the 6-h period. Interestingly, in the current study, we observed an increase in blood glucose, especially early

on and despite the reduction in FI. This would indicate that blood glucose is maintained in the face of reduced nutrient availability and this is likely due to an increase in glucose transport (Pearce et al., 2013b) or hepatic glycogenolysis. Short-term HS (4 h) in rodents has been shown to induce a reduction in blood glucose levels, without damage to the liver, showing that the liver is not responsible for the reduction in blood glucose (Leon et al., 2006). In general, the data on blood glucose changes during HS varies and lacks consistency.

In conclusion, the first 6 h of a severe heat load are critical to understanding the mechanisms behind HS. Many pathways are upregulated very early on during HS and this may provide opportunities to target these pathways for mitigation strategies. We see increased intestinal permeability, epithelial damage, expression of stress proteins, circulating endotoxin, and catabolic markers. We also have shown for the first time in pigs that HS negatively affects appetite-related neuropeptides. Understanding how a pig and other mammals first respond to HS will provide novel insights into the development of better mitigation strategies that can alleviate the negative impact HS has on performance and health.

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