Heat stress and reduced plane of nutrition decreases intestinal integrity and function in pigs


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ABSTRACT: Heat stress can compromise intestinal integrity and induce leaky gut in a variety of species. Therefore, the objectives of this study were to determine if heat stress (HS) directly or indirectly (via reduced feed intake) increases intestinal permeability in growing pigs. We hypothesized that an increased heat-load causes physiological alterations to the intestinal epithelium, resulting in compromised barrier integrity and altered intestinal function that contributes to the overall severity of HS-related illness. Crossbred gilts (n = 48, 43 ± 4 kg BW) were housed in constant climate controlled rooms in individual pens and exposed to 1) thermal neutral (TN) conditions (20°C, 35–50% humidity) with ad libitum intake, 2) HS conditions (35°C, 20–35% humidity) with ad libitum feed intake, or 3) pair-fed in TN conditions (PFTN) to eliminate confounding effects of dissimilar feed intake. Pigs were sacrificed at 1, 3, or 7 d of environmental exposure and jejunum samples were mounted into modified Ussing chambers for assessment of transepithelial electrical resistance (TER) and intestinal fluorescein isothiocyanate (FITC)-labeled lipopolysaccharide (LPS) permeability (expressed as apparent permeability coefficient, APP). Further, gene and protein markers of intestinal integrity and stress were assessed. Irrespective of d of HS exposure, plasma endotoxin levels increased 45% (P < 0.05) in HS compared with TN pigs, while jejunum TER decreased 30% (P < 0.05) and LPS APP increased 2-fold (P < 0.01). Furthermore, d 7 HS pigs tended (P = 0.06) to have increased LPS APP (41%) compared with PFTN controls. Lysozyme and alkaline phosphatase activity decreased (46 and 59%, respectively; P < 0.05) over time in HS pigs, while the immune cell marker, myeloperoxidase activity, was increased (P < 0.05) in the jejunum at d 3 and 7. These results indicate that both HS and reduced feed intake decrease intestinal integrity and increase endotoxin permeability. We hypothesize that these events may lead to increased inflammation, which might contribute to reduced pig performance during warm summer months.

Key words: Endotoxin, heat stress, intestinal integrity, pig


INTRODUCTION

Both humans and animals are adversely affected by environmental heat stress (HS; Kovats and Ebi, 2006; Renaudeau et al., 2010). Economically, it is estimated that HS results in the U.S. swine industry losing over $300 million annually, and global losses to animal agriculture are in the billions of dollars (St-Pierre et al., 2003). In growing pigs, constant HS exposure markedly increases respiration rates and body temperatures, slows body weight gains, and significantly reduces ad libitum feed intake (Pearce et al., 2013). However, when these heat-stressed pigs are directly compared with pair-fed counterparts in thermal neutral (TN)
conditions (PFTN), HS pigs gain more body weight and have distinctly different postabsorptive bioenergetic variables such as insulin (Pearce et al., 2012, 2013). These data indicate unique changes in postabsorptive metabolism that are related both to the direct effect of high thermal loads as well as reduced caloric intake and altered gastrointestinal integrity and function.

Heat-stressed mammals redistribute blood to the periphery in an attempt to maximize radiant heat dissipation, while vasoconstriction occurs in the gastrointestinal tract to reprioritize blood flow (Lambert, 2008). Consequently, the reduced blood and nutrient flow to the intestinal epithelium compromises integrity of the intestinal barrier (Yan et al., 2006). Tight junction protein complexes in the intestine are necessary for normal barrier function and their altered synthesis is implicated in certain types of stress (including HS), which can lead to increased intestinal permeability. This enhanced permeability elevates certain blood markers of endotoxemia, initiates an immune response, and activates intestinal and hepatic detoxification mechanisms (Hall et al., 2001).

Therefore, the study objective was to determine if HS directly or indirectly (via reduced feed intake) increases intestinal permeability and markers of intestinal stress in growing pigs. We hypothesized that an increased heat-load would cause physiological alterations to the intestinal epithelium, resulting in compromised barrier integrity, altered intestinal function and metabolism. Altogether, this may contribute to the overall severity of HS related illness and shifts in whole body metabolism.

MATERIALS AND METHODS

All animal procedures were approved by the Iowa State University Institutional Animal Care and Use Committee and adhered to the ethical and humane use of animals for research (IACUC# 4-10-6923S). All chemicals used for the experiment were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Animals and Experimental Design

Animals, experimental design, and nonintestinal results for this experiment have been previously described in detail (Pearce et al., 2013). Briefly, crossbred gilts \( (n = 48, 43 \pm 4 \text{ kg BW}; \text{PIC C22/C29} \times 337, \text{Carthage Veterinary Service, Carthage, IL}) \) were selected by BW and housed in individual pens (with individual feeders and waters) in one of two rooms (24 pens/room) in TN conditions \( (20 \pm 1{^\circ}\text{C}; 35 \text{ to } 50\% \text{ relative humidity}) \). Animals were allowed to acclimate to their pens for 5 d. Pigs were then assigned to one of three treatments: 1) TN conditions with ad libitum feed intake, 2) HS conditions \( (constant 35 \pm 1{^\circ}\text{C}; 20 \text{ to } 35\% \text{ relative humidity}) \) with ad libitum intake, or 3) PFTN to mirror the nutrient intake of the HS pigs as we have previously described (Pearce et al., 2013). To evaluate the temporal response to thermal treatment, pigs in the TN \( (n = 18) \) and HS \( (n = 24) \) conditions were sacrificed at d 1 \( (n = 12) \), d 3 \( (n = 12) \), and d 7 \( (n = 18) \) postinitiation of environmental treatment as previously described (Pearce et al., 2013). Pair-feeding (Rhoads et al., 2009; Pearce et al., 2013) was conducted to quantify confounding effects of dissimilar feed and nutrient intake. The PFTN pigs were only sacrificed at 7 d \( (n = 6) \) postinitiation of nutrient restriction. All animals where fed a complete diet and monitored continuously for signs of distress such as excessively high core temperature (>41{^\circ}\text{C}), weight loss, and complete loss of appetite.

Immediately before sacrifice, venous blood was collected from the jugular vein by venipuncture and centrifuged at 1300 \( \times g \) for 10 min at 4{^\circ}\text{C} to obtain serum and EDTA-plasma, which were stored at \(-20{^\circ}\text{C}\) for later analysis. At slaughter, proximal jejunum and jejunum mucosal scrapings were collected 3 m distal from the stomach, and frozen in liquid nitrogen and stored at \(-80{^\circ}\text{C}\) until later analysis. Additionally, a 20-cm fresh sample of whole jejunum was obtained and placed immediately into Krebs-Henseleit buffer (containing 25 mM NaHCO\(_3\), 120 mM NaCl, 1 mM MgSO\(_4\), 6.3 mM KCl, 2 mM CaCl, and 0.32 mM NaH\(_2\)PO\(_4\), pH 7.4) on ice under constant aeration for transport to the laboratory and mounting onto Ussing Chambers (Gabler et al., 2007, 2009; Mani et al., 2013b). Fresh jejunal segments were also immediately fixed in 10% formalin and used for histological analysis. Jejunum tissue was chosen due to its importance in nutrient absorption, high blood flow, and sensitivity to both endotoxemia and hypoxia (Maier et al., 2009).

Intestinal Histology

Whole jejunal samples fixed in formalin were sent to the Iowa State University Veterinary Diagnostic Laboratory for sectioning and hematoxylin and eosin staining of intestinal tissues and structures. Using a microscope (DMI3000 B Inverted Microscope, Leica Microsystems, Bannockburn, IL) with an attached camera (12-bit QICAM Fast 1394, QImaging, Surrey, BC, Canada), pictures were obtained of 10 villi and 10 crypts per sample section across three sections. Each image was measured for villus height and crypt depth. Finally, the averages of 30 villi and crypts were calculated and reported as one number per pig. Images of individual villi and crypts were obtained using Q-capture Pro 6.0 (QImaging, Surrey, BC) and measurements were taken using Image-Pro Plus 7.0 (Media Cybernetics, Bethesda, MD).
**Ex Vivo Intestinal Integrity and Lipopolysaccharide Permeability**

Fresh segments of proximal jejunum were collected and mounted into modified Ussing chambers (Physiological Instruments, San Diego, CA) for determination of intestinal integrity and endotoxin or labeled lipopolysaccharide (LPS) transport. One representative tissue sample from each pig was pinned and placed vertically into the chambers connected to dual channel current and voltage electrodes submerged in 3% noble agar and filled with 3 M KCl for electrical conductance. Each segment was bathed in 4 mL of Krebs-Henseleit buffer on both serosal and mucosal sides, and tissue was provided with a constant O$_2$-CO$_2$ mixture. Individual segments were clamped at a voltage of 0 mV and transepithelial electrical resistance (TER) determined over 30 min and calculated by averaging the current during the first 10 min of tissue stabilization (Gabler et al., 2007).

Thereafter, jejunum segments were also assessed for endotoxin permeability using fluorescein isothiocyanate (FITC)-LPS (from *Escherichia coli* 055:B5) as previously described (Tomita et al., 2004; Mani et al., 2013b). Briefly, after 30 min of tissue stabilization in modified Ussing chambers, 20 μg/mL of FITC-LPS was added to the mucosal side and media samples from both the mucosal and serosal chambers were obtained every 20 min for 120 min and read in a fluorescence spectrophotometer at 495 nm. An apparent permeability coefficient (APP) for FITC-LPS across the jejunum was then calculated (Tomita et al., 2004; Mani et al., 2013b).

**Circulating Endotoxin Assay**

Plasma 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 (rFC) endotoxin assay with a 1/1000 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 again after 4 min on a Synergy 4 microplate reader (Bio-Tek, Winosoki, VT). The assay was performed in 96-well plates at 405 nm at t = 0 and again after 4 min on a Synergy 4 microplate reader (Bio-Tek, Winosoki, VT).

Myeloperoxidase (MPO) activity of whole jejunum was measured using a modified method by Suzuki et al. (1983). Tissue samples were homogenized in 0.5% hexadecyltri-methylammonium bromide (HTAB) in potassium phosphate buffer (PPB, pH 6.0) and then freeze-thawed and vortexed three times. Samples were then centrifuged for 15 min at 10,000 × g and 4°C. The resulting supernatant was transferred to a new tube and the remaining pellet was resuspended in 500 μL of 0.5% PPB + HTAB. The resuspended pellet was freeze-thawed and homogenized 2 times, and 500 μL was transferred to a new tube. Samples were then centrifuged again at 10,000 × g for 15 min at 4°C and the supernatant was collected. The final supernatant was mixed with o-dianisidine dihydrochloride and 0.005% hydrogen peroxide. One unit of MPO activity was expressed as the amount of MPO needed to degrade 1 μmol of hydrogen peroxide · min$^{-1}$ · mL$^{-1}$. Absorbance was read at 460 nm for 10 min reaction time, and absorbance was calculated on a milliliter sample per milligram tissue basis.

Jejunum mucosal tissue samples were extracted using T-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) supplemented with protease (Roche, Indianapolis, IN) inhibitor tablets and 12 mM butylated hydroxytoluene, and lysates were centrifuged at 10,000 × g for 5 min at 4°C. Protein concentration of the jejunum lysates was determined using the Bicinchoninic Acid Assay method. Equal amounts of jejunal tissue protein (100 μg) were analyzed for interleukin 8 (IL-8) concentration us-
ing a porcine-specific ELISA (DuoSet Porcine IL-8, catalog number DY535, R&D systems, Minneapolis, MN) per the manufacturer’s instructions. Data was expressed on a per-unit protein basis. The assay detection range was 0 to 8000 pg/mL, and the inter- and intraassay coefficients were 10.8 and 12.9%, respectively.

For the determination of jejunal 4-hydroxynonenal (4-HNE) adducts, slot blot analysis was performed according to previously published procedures used to evaluate the effect of inflammation on tissue 4-HNE adduct levels (Yin et al., 2009). Protein lysates (15 μg) were blotted onto nitrocellulose membranes in duplicate using a slot blot apparatus. The blots were stained with Ponceau S to visualize protein transfer, and the membranes were incubated overnight at 4°C with the 4-HNE goat antirabbit antibody (AB5605, Millipore, Temecula, CA) at a dilution of 1:5000. A goat antirabbit IgG conjugated to horseradish peroxidase (HRP, Pierce) and a chemiluminescent detection kit were used along with a Kodak Image Pro 4000 mm imaging system and software (Rochester, NY) to visualize and quantify the reaction complexes.

Mucosal scrapings were also probed for total glutathione concentration following homogenization in ice-cold MES buffer containing 1 mM EDTA and centrifuged at 10,000 × g at 4°C for 15 min. The resulting supernatant was deproteinized by adding an equal volume of 10% metaphosphoric acid and centrifugation at 2000 × g for 2 min at 4°C. Total glutathione concentration in the deproteinized lysate was determined using a colorimetric glutathione kit (Cayman Chemical, Ann Arbor, MI) and expressed on a per-unit protein basis.

Plasma haptoglobin from d 7 samples was analyzed using a commercially available ELISA (ALPCO diagnostics, Salem, NH) as a marker of systemic inflammatory stress response. Briefly, samples were added to wells adsorbed with antiporcine haptoglobin antibodies. After washing, HRP-conjugated antihaptoglobin antibodies were added to the plate. After another washing, the HRP was assayed by the addition of the chromogenic substrate 3, 3´, 5, 5´-tetramethylbenzidine (TMB) and the absorbance was measured at 450 nm. The quantity of haptoglobin in the test samples was interpolated from the standard curve constructed from the standards and corrected for sample dilution. The haptoglobin assay detection range was 25 to 400 ng/mL, and the intraassay coefficient was 4.9%.

**Jejunum Na⁺/K⁺ ATPase Activity**

Ileal mucosal scrapings were homogenized in sucrose buffer (pH 7.4) consisting of 50 mM sucrose, 1 mM Na₂EDTA, and 20 mM tris base, and centrifuged at 1000 × g for 10 min at 4°C for protein extraction. Protein extracts were separated into 5 aliquots: two for water, two for ouabain, and one for BCA protein analysis. Proteins with either MQ H₂O or 20 mM ouabain were preincubated for 15 min with Na⁺/K⁺ ATPase reaction buffer (pH 7.0; 2000 mM NaCl, 100 mM KCl, 50 mM MgCl₂ and 250 mM HEPES) and then incubated for 45 min after addition of fresh 105 mM ATP to start the reaction. After 45 min the reaction was terminated using ice-cold 50% trichloroacetic acid. Samples were centrifuged at 1500 × g for 10 min at 4°C to obtain the final product which was present in the supernatant (Fuller et al., 2003). Lastly, samples were analyzed for the presence of inorganic phosphate (P_i) using the Molybdenum blue method (Ueda and Wada, 1970) and assessed in triplicate at a wavelength of 400 nm using a Synergy 4 microplate reader (Bio-Tek, Winooski, VT). Specific Na⁺/K⁺ ATPase activity was determined by the difference in P_i production from ATP in the presence of absence of ouabain (specific Na⁺/K⁺ ATPase inhibitor). Unspecific phosphate hydrolysis was calculated by measuring liberated P_i in the absence of protein suspension and expressed on a per-unit time and protein basis.

**RNA Isolation and Quantitative PCR**

Total RNA was isolated from tissue samples using a commercially available kit (RNeasy fibrous tissue mini kit, Qiagen, Valencia, CA). Total RNA was quantified by measuring the absorbance at 260 nm using a spectrophotometer (ND-100, NanoDrop Technologies, Rockland, DE) and purity was assessed by determining the ratio of the absorbance at 260 nm and 280 nm (NanoDrop). All samples had 260/280 nm ratios above 1.8 and the integrity of the RNA preparations was also verified by visualization of the 18S and 28S ribosomal bands stained with SYBR Safe DNA gel stain (Life Technologies, Carlsbad, CA) after electrophoresis on 2% agarose gels. Total RNA (1 μg) was transcribed in a reaction combining genomic DNA elimination using a commercially available cDNA synthesis kit (Quantitect reverse transcription kit, Qiagen, Valencia, CA). cDNA was quantified using NanoDrop and used for real-time quantitative PCR reaction. Amplification was performed in a total volume of 25 μL containing 2× quantitect SYBR Green PCR master mix (iQ SYBR green supermix, Bio-Rad, Hercules, CA), forward and reverse primers (0.3 μM), and 200 ng of cDNA. Primer sequences for target genes and the housekeeper gene are listed in Table 1. The cycling conditions included an initial 15 min denaturation step at 95°C, and then reactions were cycled 50 times under the following parameters: 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Optical detection was performed at 72°C. At the end of the PCR run, melt curve analysis was conducted to validate the specificity of the primers. A nontemplate control was run with every assay and all determinations.
were performed in triplicate. The mRNA abundance values for each sample were normalized to RPL32 according to the $2^{ΔΔCT}$ method (Livak and Schmittgen, 2001).

**Data Analysis**

Data were evaluated using two distinct models statistically analyzed using the PROC MIXED procedure of SAS v. 9.2 (SAS Inst. Inc., Cary, NC). Pig was considered our experimental unit to assess the effects of HS on the pig’s physiology over time. As expected, there were no differences within the TN treatment across time (d 0, 3, and 7). Therefore, the first statistical model considered these pigs as zero or no HS and the fixed effect of time (days) was used to compare TN (d 0) with d 1, 3, and 7 of HS. These data were further analyzed for linear and quadratic contrasts to better understand the type of changes in our parameters measured over time. By design the second statistical model tested for differences between TN, PFTN, and HS treatments as fixed effects only at d 7 (Pearce et al., 2013). All data are reported as least squares means ± SEM and considered significant if $P < 0.05$ and a trend if $P < 0.10$.

**RESULTS**

**Effect of 0, 1, 3, and 7 d of Heat Stress on Markers of Intestinal Integrity and Function**

During the 7 d of HS, jejunum TER (Fig. 1) linearly decreased ($P = 0.02$). Additionally, an opposite quadratic response ($P = 0.023$) was observed for jejunum LPS permeability over the 7-d period (Fig. 1). A 118% increase ($P < 0.05$) in FITC-LPS APP by d 3, but only a 52% increase by d 7 ($P > 0.05$) was also observed. Blood endotoxin (Fig. 2) was equally elevated at all HS d (1, 3, and 7), compared with TN d 0 control pigs ($P < 0.05$). Furthermore, these data were supported by a linear and quadratic ($P < 0.10$) increase in plasma endotoxin over the 7-d period (Fig. 2). Jejunum morphology was also significantly affected by prolonged heat exposure (Table 2). Villus height and villus:crypt ratio were decreased (in a linear and quadratic manner, $P < 0.05$) at d 1, 3, and 7, by up to 23% compared with the d 0 TN pigs. Although the variation was small, crypt depth increased over the first 3 d of HS, and then decreased at d 7 compared with d 0 TN pigs ($P < 0.05$).

Markers of intestinal stress, inflammation, and function were assessed at d 0, 1, 3, and 7 of HS (Table 3). Interestingly, jejunum Na$^+/K^+$ ATPase activity and the

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**Table 1. Primers used**

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<th>Gene</th>
<th>Sense (5’ to 3’)</th>
<th>Antisense (5’ to 3’)</th>
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<td>GAG CTG AGC GTT AGG AGC</td>
<td>GGG CAG CTT GTA TTT TCG AG</td>
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<td>HIF-1α</td>
<td>CGT GCG ACC ATG AGG AAA</td>
<td>TGC AGT GAA GTA CTT TCC ATG</td>
</tr>
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<td>MLCK</td>
<td>CCA ACC AGC CCG CTG AAT GC</td>
<td>AGC GAC GCC TCT AAG GAA AGC</td>
</tr>
<tr>
<td>Occludin</td>
<td>ATC CTG GTG ATG GTG TT</td>
<td>ACT GGT TGC AGG GGC CAT AG</td>
</tr>
<tr>
<td>ZO-1</td>
<td>AAT TAT CCC ACA GGG AGC</td>
<td>AGG GTT TCA CCT TCC TTA TC</td>
</tr>
<tr>
<td>Claudin 3</td>
<td>CAT CGG CAG CAG CAT TAT C</td>
<td>ACA CTT TGC ACT GCA TCT GG</td>
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<td>RPL32</td>
<td>TGG AAG AGA GTG TGT GAG CAA</td>
<td>CGG AAG TTT CTG GTA CAC AAT GTA</td>
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**Figure 1.** Heat stress alters ex vivo jejunum lipopolysaccharide permeability (circles) and transepithelial resistance (squares) in pigs. Intestines were isolated from growing pigs after exposure to thermal neutral conditions (d 0, 20°C) or 1, 3 or 7 d of constant heat stress (35°C) and mounted into modified Ussing chambers. Data are arbitrary means ± SEM D 0 n = 18, d 1 and 3 n = 6, and d 7 n = 12. $a,b$Means without a common letter differ ($P < 0.05$). Labeled lipopolysaccharide (LPS) permeability contrast $P$-values: Linear (0.06) and Quadratic (0.023). Transepithelial resistance contrast $P$-values: Linear (0.022) and Quadratic (0.38).

**Figure 2.** Heat stress augments blood endotoxin concentrations in pigs over a 7-d period. Pigs were exposed to either thermal neutral conditions (d 0, 20°C) or constant heat stress (35°C) for 1, 3, or 7 d. Data are means ± SEM; d 0 n = 18; d 1 and 3 n = 6, and d 7 n = 12. $a,b$Means without a common letter differ ($P < 0.05$).
oxidative stress marker 4-HNE increased within the first 24 h of HS ($P < 0.05$). However, concentrations of the antioxidant glutathione tended to decrease by 26% ($P = 0.062$) by d 7 in a linear fashion ($P = 0.016$). There were no differences observed over time in the jejunum in regards to the cytokine and chemoattractant IL-8 ($P > 0.05$). The neutrophil infiltration marker, MPO activity, significantly increased in the jejunum at d 3 and 7 ($P < 0.01$). However, concentrations of the antioxidant glutathione tended to decrease by 26% ($P = 0.062$) by d 7 in a linear fashion ($P = 0.016$). There were no differences observed over time in the jejunum in regards to the cytokine and chemoattractant IL-8 ($P > 0.05$). The neutrophil infiltration marker, MPO activity, significantly increased in the jejunum at d 3 and 7 ($P < 0.01$). However, concentrations of the antioxidant glutathione tended to decrease by 26% ($P = 0.062$) by d 7 in a linear fashion ($P = 0.016$). There were no differences detected in TN, HS, and PFTN pigs after 7 d of treatment for TER ($P > 0.10$, Table 5). However, ex vivo jejunum LPS permeability (Table 5) increased due to HS (240%, $P = 0.045$). While not statistically different, PFTN pigs also experienced an increase (170%) in LPS permeability compared with TN controls. There were no differences between treatments in plasma endotoxin after 7 d. Interestingly, compared with TN pigs, blood concentrations of haptoglobin, an acute phase protein, were elevated by 7 d of HS (171%, $P < 0.05$). However, this was not observed in the PFTN pigs, which were not different from their TN counterparts ($P > 0.10$, Table 5).

Compared with TN controls, 7 d of HS ($P < 0.05$) reduced villus height (Fig. 3A) and villus height:crypt depth ratio (Fig. 3C). Additionally, both HS and PFTN reduced jejunum crypt depth by 5 to 18%, respectively, compared with TN pigs (Fig. 3B, $P < 0.05$). The PFTN conditions did not alter pig jejunum villus height and villus height:crypt ratio compared with TN controls (Fig. 3A, 3C). However, compared with both the TN and HS pigs, PFTN pigs had shorter crypt depths ($P < 0.05$, Fig. 3B).

By d 7, the HS pigs had elevated levels of the lipid peroxidation marker, 4-HNE, compared with TN and PFTN pigs ($P < 0.05$, Fig. 4A). As expected, HS resulted in a 50% increase in 4-HNE ($P < 0.05$). Interestingly, both HS and PFTN pigs had lower intestinal glutathione concentrations (36 and 61%, respectively)

### Table 2. The effect of constant heat stress on jejunum morphology

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Day of heat stress$^1$</th>
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<tr>
<td></td>
<td>0</td>
<td>1</td>
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<tr>
<td>Villus Height, µm</td>
<td>499$^a$ ± 7.8</td>
<td>426$^b$ ± 12.9</td>
</tr>
<tr>
<td>Crypt Depth, µm</td>
<td>289$^a$ ± 3.1</td>
<td>304$^b$ ± 5.1</td>
</tr>
<tr>
<td>Villus:Crypt ratio</td>
<td>1.79$^a$ ± 0.02</td>
<td>1.43$^b$ ± 0.04</td>
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</tbody>
</table>

$^a,b$Means without a common letter differ ($P < 0.05$).

$^1$Pigs were exposed to either thermal neutral conditions (d 0, 20°C) or 1, 3, or 7 d of constant heat stress (35°C). Mean ± SEM; d 0 n = 18, d 1 and 3 n = 6, and d 7 n = 12.

### Table 3. Effects of heat stress on intestinal stress, inflammation, and function markers

<table>
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<tr>
<th>Parameter</th>
<th>Day of heat stress$^1$</th>
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</thead>
<tbody>
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<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Na+/K+ ATPase, µmol Pi ∙ min$^{-1}$ ∙ mg$^{-1}$ protein</td>
<td>136.9$^a$ ± 28.88</td>
<td>352.9$^b$ ± 45.24</td>
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<td>4-Hydroxynonenal, arbitrary units</td>
<td>17926$^a$ ± 1376</td>
<td>22211$^b$ ± 2384</td>
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<tr>
<td>Glutathione, µg/g protein</td>
<td>0.72$^a$ ± 0.068</td>
<td>0.87$^a$ ± 0.120</td>
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<td>Interleukin-8, µg/g protein</td>
<td>1.07$^a$ ± 0.147</td>
<td>1.42$^b$ ± 0.263</td>
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<tr>
<td>Myeloperoxidase, mU/mg protein</td>
<td>1.75$^a$ ± 0.390</td>
<td>2.23$^a$ ± 0.670</td>
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<tr>
<td>Lysozyme, U/mg protein</td>
<td>11.75$^a$ ± 0.820</td>
<td>11.27$^b$ ± 1.420</td>
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<td>Alkaline phosphatase, mU/mg protein</td>
<td>157.7$^a$ ± 16.90</td>
<td>140.7$^a$ ± 29.26</td>
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</table>

$^a,b$Means without a common letter differ ($P < 0.05$).

$^x,y,z$Means without a common letter tend to differ ($P < 0.10$).

$^1$Pigs were exposed to either thermal neutral conditions (d 0, 20°C), or 1, 3 or 7 d of constant heat stress (35°C). Mean ± SEM; d 0 n = 18, d 1 and 3 n = 6, and d 7 n = 12.

### Constant Heat Stress and Reduced Feed Intake on Intestinal Integrity and Function

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Acknowledgments:
Table 4. Heat stress induces changes in jejunum stress and integrity mRNA abundance in pigs

<table>
<thead>
<tr>
<th>Parameter</th>
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<tr>
<td>Occludin, fold change(^2)</td>
<td>1.7 ± 0.45</td>
<td>2.3 ± 0.78</td>
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<td>ZO-1, fold change(^2)</td>
<td>1.3 ± 0.24</td>
<td>0.8 ± 0.43</td>
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<td>Claudin, fold change(^2)</td>
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<td>0.5 ± 0.62</td>
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<td>HSP27, fold change(^2)</td>
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<td>4.9 ± 1.82</td>
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<td>HIF-1α, fold change(^2)</td>
<td>1.1 ± 0.93</td>
<td>1.4 ± 1.61</td>
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<td>MLCK, fold change(^2)</td>
<td>1.1 ± 0.25</td>
<td>1.2 ± 0.40</td>
</tr>
</tbody>
</table>

\(^{a,b,c}\) Means without a common letter differ (P < 0.05).
\(^{x,y,z}\) Means without a common letter tend to differ (P < 0.10).

\(^1\) Pigs were exposed to either thermal neutral conditions (d 0, 20°C) or 1, 3, or 7 d of constant heat stress (35°C). Mean ± SEM; n = 18, d 1 and 3 n = 6, and d 7 n = 12.

\(^2\) mRNA abundance (fold change) values for each sample were normalized to RPL32 according to the 2\(^{ΔΔCT}\) method.

\(^3\) Hypoxia inducible factor 1 alpha.

\(^4\) Myosin light chain kinase.

\(^5\) Heat shock protein 27.

\(^6\) Zonula occludin 1.

Compared with TN pigs (Fig. 4B, P < 0.05). Concentrations of intestinal IL-8 (Fig. 4C) were not affected by pair-feeding, but were decreased 36% due to HS (P < 0.05). Heat stress treatment increased intestinal MPO activity (Fig. 4D), a measure of neutrophil infiltration and inflammation. However, HS intestinal lysosome activity was lower compared with PFTN pigs (P < 0.05), but not TN control pigs (Fig. 4E). Alkaline phosphatase activity (Fig. 4F) was not affected by pair-feeding, but was decreased 65%, due to HS (P < 0.05). After 7 d of experimental treatment, compared with the TN control, jejunum Na\(^+\)/K\(^+\) ATPase activity (P < 0.05) decreased in the PFTN group, and tended (P < 0.10) to be attenuated with HS (143, 77, and 108 µmol P i –1 mg protein –1 h –1 , respectively).

Tight junction occludin mRNA abundance (Fig. 5) tended to be increased by approximately 1- to 2-fold in the jejunum of HS pigs compared with TN pigs (P < 0.10), and was statistically different for ZO-1 (P < 0.05) and claudin 3 (P < 0.05) abundance. However, there was no difference between HS and PFTN claudin 3 mRNA abundance. Heat shock protein 27 and HIF-1α mRNA abundance did not differ between treatments on d 7 (Fig. 5D and 5E, respectively). Interestingly, at d 7, MLCK mRNA abundance was lower in HS and PFTN (P < 0.05, Fig. 5F) compared with the TN pigs, however, HS and PFTN pigs were not different.

**DISCUSSION**

Heat stress disrupts intestinal tight junctions and increases intestinal permeability in rodents and humans (Hall et al., 2001; Dokladny et al., 2006), but whether this is directly caused by heat or indirectly mediated by reduced nutrient intake is not clear. Therefore, the objectives of this study were to determine if HS increases intestinal permeability directly or indirectly (mediated by reduced feed intake) in growing pigs. We report herein, both a feed intake and HS by time dependent regulation of intestinal integrity and function. As environmental heat-load increases, blood is diverted to the skin (via coordinated peripheral vasodilatation and gastrointestinal tract vasoconstriction), orchestrating the partitioning of blood away from the splanchnic bed (Hall et al., 1999). Consequently, the intestinal epithelium can become hypoxic, acidic, ATP depleted, experience oxidative or nitrosative stress, and ultimately, apoptosis can occur (Yan et al., 2006). These insults can damage enterocytes, increase permeability, and eventually lead to endotoxemia, inflammation, and organ damage (Lambert, 2004). In support of this, we observed an acute increase in HIF-1α

Table 5. Effects of 7 d of heat stress or feed intake restriction on intestinal ex vivo intestinal integrity and blood stress markers

<table>
<thead>
<tr>
<th>Parameter</th>
<th>TN(^2)</th>
<th>HS(^2)</th>
<th>PFTN(^2)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transepithelial resistance, arbitrary units</td>
<td>1.0 ± 0.20</td>
<td>0.8 ± 0.13</td>
<td>0.8 ± 0.18</td>
<td>0.72</td>
</tr>
<tr>
<td>LPS permeability (APP), arbitrary units(^3)</td>
<td>1.0 ± 0.43</td>
<td>2.4 ± 0.29</td>
<td>1.7 ± 0.39</td>
<td>0.045</td>
</tr>
<tr>
<td>Blood endotoxin, arbitrary units</td>
<td>27 ± 4.4</td>
<td>35 ± 3.1</td>
<td>34 ± 4.8</td>
<td>0.35</td>
</tr>
<tr>
<td>Blood haptoglobin, mg/mL</td>
<td>0.7 ± 0.25</td>
<td>1.9 ± 0.29</td>
<td>0.5 ± 0.25</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

\(^{a,b,c}\) Means without a common letter differ (P < 0.05).

\(^1\) Pigs were exposed to thermal neutral (TN) conditions (20°C), constant heat stress (HS) conditions (35°C), or pair-fed to the HS feed intake under TN conditions (PFTN) for 7 d.

\(^2\) Mean ± SEM. TN n = 6, HS n = 12, and PFTN n = 6.

\(^3\) LPS, labeled lipopolysaccharide; APP, apparent permeability coefficient.
mRNA abundance, 4-HNE, and a reduction in glutathione due to HS. Increases in 4-HNE has been related to decreased feed efficiency and altered metabolism in broilers (Bottje and Harrison, 1985; Bottje and Carstens, 2009). Additionally, we observed that HS, but not pair-feeding, significantly increased Na\(^+/K^+\) ATPase activity in the intestinal tract. Ion pumps are energetically expensive and involved in the active pumping of ions across the plasma membrane at the cost of ATP hydrolysis. Thus, in excess of 20% of the energy expenditure in skeletal muscle, liver, and intestines is thought to be attributed to Na\(^+/K^+\) transport and 10% to Ca\(^{2+}\) transport (Milligan and McBride, 1985). We speculate that these ion pumps are upregulated during HS to help maintain osmotic homeostasis in the intestinal tract. If ion balance and osmolality are not tightly regulated, perturbation of enzyme function, protein structure, membrane integrity, and water retention may result (Craig, 2004; Street et al., 2010).

Morphological changes in the small intestine were also observed in our study; however, HS effects on morphology may be varied. Pigs heat-stressed to 40°C observed decreased villus height and crypt depth in jejunum, as well as duodenum (Yu et al., 2010). Similarly, HS resulted in shorter villus height in duodenum and jejunum tissue as well as shallower crypt depth (Liu et al., 2009). Shortened height of intestinal villi and crypts indicates damage to the intestinal epithelium. Damage to the epithelium (i.e., epithelial sloughing and necrosis) is thought to contribute largely to increased permeability (Lambert et al., 2002). This damage appears to occur before other organ damage because reduced blood flow affects the intestine first. Damage to the intestinal epithelium may also affect digestion and absorption of nutrients (Liu et al., 2009). Interestingly, nutrient restriction alone (similar to the HS and PFTN pigs) can lead to alterations in intestinal function, transport, morphology, and may increase the risk of developing bacterial sepsis (Ferraris and Carey, 2000). Therefore the effects seen during HS may be confounded and exacerbated by the dramatic reduction in feed intake. Welsh and co-workers (1998) suggested that increased permeability to macronutrients in moderately and severely malnourished humans may maximize nutrient absorption, but this may also increases the chance of bacterial translocation.

Heat stress has been shown to modulate intestinal integrity (Lambert, 2004; Dokladny et al., 2008). The intestinal epithelium is comprised of adhesion molecules, gap and tight junction proteins which form the protective barrier that aids in absorption and transport of nutrients while preventing translocation of potentially harmful molecules. Epithelial cells also consist of an actin cytoskeleton, which forms a large part of the epithelial barrier. Regulated by MLCK activation, contraction of the actin cytoskeleton helps maintain cell motility, leading to the opening of tight junctions and increased intestinal permeability (Yang et al., 2007; Lambert, 2009). Our data indicates a cyclical effect of HS on MLCK and tight junction gene abundance, as it is rapidly upregulated in acute HS, but then decreases drastically by d 3. In vitro, HS also causes alterations in the distribution of the protein ZO-1, such as diffusion to cytosol (Ikari et al., 2005). Similarly, ZO-1 protein expression is also de-
creased, indicating tight junction disruption (Dokladny et al., 2006). Our data is consistent with previous observations in which HS has also been shown to upregulate occludin gene expression (Dokladny et al., 2008), which is an important tight junction protein for maintaining barrier function, and increased expression may indicate a protective response related to gastrointestinal epithelial restitution. Following intestinal stress, the acute phase response is important in improving intestinal epithelial cell wound healing and restitution (Richter et al., 2012).

As changes in tight junction proteins and MLCK are related to changes in intestinal permeability and resistance, we measured jejunal TER as a proxy of how tight or leaky the intestinal epithelium was in this study. We have previously reported ileum and colon FITC-dextran (4 kDa) macromolecule permeability to be increased in pigs due to HS (Pearce et al., 2012). Consistent with in vitro (Dokladny et al., 2006) and in vivo rodent data (Prosser et al., 2004), over time HS reduced intestinal TER in the current study. This linear TER reduction in HS pigs is also supported by the increased LPS APP and blood endotoxin data. Increased APP is a qualitative measure of FITC-LPS intestinal permeability and is another indicator of leakiness. Although we suspect paracellular transport, LPS may be transported across the intestinal epithelium in active, passive, transcellular, or receptor mediated processes (Mani et al., 2012, 2013b).

Many of the effects of HS on intestinal integrity parameters appear to be directly mediated by reduced feed intake as PFTN control variables were similar to HS pigs. Consequently, many biological effects at the intestine purportedly caused by HS (Hall et al., 2001; Lambert, 2004; Leon, 2007) appear to be indirect (mediated by reduced feed intake) effects of environmentally induced hyperthermia. To our knowledge, this is the first trial to evaluate the effects of HS on intestinal permeability while experimentally controlling for reduced nutrient intake in pigs.

The presence of higher blood endotoxin has the potential to contribute to the development of an inflammatory state and reduce growth potential. In our study, serum endotoxin and the acute phase protein, haptoglobin, tended to be higher in HS animals. The blood endotoxin data is consistent with heat-stressed rodent models (Hall et al., 2001; Lim et al., 2007), chickens (Cronje, 2007) as well as human heat-stroke patients (Bouchama et al., 1991). This is a function of both increased intestinal permeability of LPS and reduced LPS detoxification, neutralization or clearance from the body. Intestinal alkaline phosphatase and lysozyme activities were reduced due to HS. We believe that this is contributing to the increased inflammatory and LPS responses observed during HS. Compromised mucosal alkaline phosphatase and lysozyme activity may increase transmucosal passage of bacteria and enteric pathogens, and reduce the protection against LPS-induced inflammation (Takada et al., 1994; Lackeyram et al., 2010; Lallès, 2010; Mani et al., 2013a). Although we observed no increased in intestinal cytokines, MPO activity was significantly increased. Both of these markers are commonly used for assessing intestinal inflammation and neutrophil infiltration (Suzuki et al., 1983).
The changes we have reported herein in intestinal physiology and blood parameters may partially explain the major changes in body temperature indices and production variables we have previously reported in these pigs (Pearce et al., 2013). We previously reported that HS pigs had a marked and sustained increase in rectal temperatures (1.5°C) and respiration rates (2-fold) compared with TN and PFTN pigs over the 7-d test period. Furthermore, HS pigs consumed less feed starting from d 1 of HS and remained constantly reduced over the 7-d period (46%) compared with TN pigs. By design, the PFTN pigs feed intake mirrored that of the HS pigs. Pigs in TN conditions gained body weight (1.14 kg/d) throughout the experiment, while the HS pigs initially lost 2.7 kg of BW at d 1, but gained 0.03 and 1.65 kg cumulatively by d 3 and 7, respectively compared with initial body weights. The PFTN pigs had lost 2.47 kg of BW by d 7. These phenotypic changes may be related to a decrease in intestinal integrity and an increase in endotoxin permeability. Interestingly, our data suggests that feed intake restriction may orchestrate some of this stress-related response as explained by our PFTN treatment.

In conclusion, intestinal integrity, function, and metabolism can be compromised due to prolonged HS. This may partially be attributed to the reduced nutrient intake, as seen in the PFTN pigs, or as a consequence of potential changes in digesta flow rates and motility as a result of this reduced feed intake. Altogether, these data may help explain how HS directly and indirectly (via reduced feed intake) affected intestinal physiology, and post-absorptive metabolism and growth performance parameters observed in our previous study (Pearce et al., 2013).

LITERATURE CITED


Heat stress and intestinal integrity


