Dietary organic zinc attenuates heat stress–induced changes in pig intestinal integrity and metabolism[1,2]

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ABSTRACT: Dietary zinc (inorganic and organic or zinc AA complex forms) is essential for normal intestinal barrier function and regeneration of intestinal epithelium. Given that heat stress (HS) exposure can negatively affect intestinal integrity and caloric intake, possible nutritional mitigation strategies are needed to improve health, performance, and well-being. Therefore, our objective was to evaluate 2 dietary zinc sources and reduced caloric intake on intestinal integrity in growing pigs subjected to 12 h of HS. A total of 36 pigs were fed 1 of 2 diets: 1) a control diet (CON; 120 mg/kg of zinc from zinc sulfate) or 2) 60 mg/kg from zinc sulfate and 60 mg/kg from zinc AA complex (ZnAA). After 17 d, the CON pigs were then exposed to thermal neutral (TN) conditions with ad libitum intake (TN-CON), HS (37°C) with ad libitum intake (HS-CON), or pair-fed to HS intake under TN conditions (PFTN); the ZnAA pigs were exposed to only HS (HS-ZnAA). All pigs were sacrificed after 12 h of environmental exposure, and blood and tissue bioenergetics stress markers and ex vivo ileum and colon integrity were assessed. Compared with TN-CON, HS significantly ($P < 0.05$) increased rectal temperatures and respiration rates. Ileum villus and crypt morphology was reduced by both pair-feeding and HS. Both PFTN and HS-CON pigs also had reduced ileum integrity (dextran flux and transepithelial resistance) compared with the TN-CON pigs. However, ZnAA tended to mitigate the HS-induced changes in ileum integrity. Ileum mucin 2 protein abundance was increased due to HS and pair-feeding. Colonic integrity did not differ due to HS or PFTN treatments. Compared with the HS-CON, HS-ZnAA pigs tended to have reduced blood endotoxin concentrations. In conclusion, HS and reduced feed intake compromised intestinal integrity in pigs, and zinc AA complex source mitigates some of these negative effects.

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

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INTRODUCTION

Swine are negatively affected by high ambient temperatures (Renaudeau et al., 2011; Song et al., 2011; Pearce et al., 2013a,b; Johnson et al., 2015). We have previously reported that both heat stress (**HS**) and reduced feed intake decrease intestinal integrity and increase endotoxin permeability in growing pigs (Pearce et al., 2013c, 2014). Identifying possible nutritional mitigation strategies may provide options for animal agriculture. One possibility is the use of zinc, as increasing supplemental dietary zinc in children has been shown to ameliorate changes in intestinal permeability (Alam et al., 1994).

Zinc is an activator of many enzymes and therefore influences the acid–base balance, immune competence, and basic cellular functions (Golding, 2002; Takkar, 2011). It is also essential for normal intestinal barrier function and the regeneration of injured intestinal epithelial tissue (Alam et al., 1994).

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Furthermore, dietary zinc has been shown to improve and prevent reductions in intestinal integrity during malnutrition (Rodriguez et al., 1996), chronic inflammatory bowel diseases (Sturniolo et al., 2001), HS (Sanz Fernandez et al., 2014), and infectious diarrhea (Alam et al., 1994) and reduces intestinal permeability of piglets during weaning (Zhang and Guo, 2009). Based on these biological functions, zinc may be an attractive feed additive for alleviating the negative effects of intestinal-related stresses.

Therefore, our objective was to evaluate whether zinc AA complex could mitigate changes in HS-induced intestinal integrity, permeability, and injury in a severe short-term HS pig model. Additionally, using a pair-fed thermal neutral (**TN**) treatment, this study also examined the impact of reduced caloric intake independent of heat on intestinal function and integrity. We hypothesized that feeding a more bioavailable organic zinc source would improve intestinal barrier function during a severe heat load.

MATERIALS AND METHODS

Animals, Diets, 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). Thirty-two crossbred gilts (64 ± 2.9 kg BW) were assigned to 1 of 2 dietary treatments at the Iowa State University Swine Nutrition Farm (Ames, IA) based on initial BW. All pigs were fed an isoenergetic and isonitrogenous diet formulated to meet or exceed the predicted requirements (NRC, 1998) for energy, essential AA, protein, minerals, and vitamins (Table 1). Twenty-four pigs were fed a control diet (**CON**) formulated to provide 120 mg/kg of supplemental zinc, the form of which was zinc sulfate (Sigma-Aldrich, St. Louis, MO) and 8 pigs received zinc AA complex (**ZnAA**) formulated to provide 120 mg/kg total supplemental zinc, of which 60 mg/kg was provided by zinc sulfate and 60 mg/kg supplied by organic zinc (zinc AA complex; AvailaZinc 100; Zinpro Corporation, Eden Prairie, MN). This is a proprietary blend of AA bound to a mineral molecule.

All pigs were fed their respective diets ad libitum for approximately 17 d before the start of the experimental challenge period. During this time, all animals were group housed (4 pigs/pen) according to their dietary treatment and reared under TN conditions (21–24°C and 50–70% humidity). Thereafter, pigs were moved to individual pens (TN conditions) and allowed 3 d to acclimate before their respective 12-h environmental challenges. Pigs where allotted to 1 of 4 environmental treatments: 1) HS with ad libitum intake (**HS-CON**; 37°C and approximately 40% humidity; $n = 8$), 2) the ZnAA diet ad libitum under HS (**HS-ZnAA**; 37°C and approximately 40% humidity; $n = 8$), 3) TN conditions (21°C and approximately 70% humidity) with ad libitum intake (**TN-CON**; $n = 8$), or 4) pair-feeding to their HS-CON counterparts and exposed to TN conditions (**PFTN-CON**; $n = 8$). The feed intake (**FI**) for those pair-fed to HS intake under TN conditions (**PFTN**) pigs was estimated based on their TN ad libitum FI and the reduction expected from HS conditions based on our previous experiments (Pearce et al., 2013a,b; Sanz Fernandez et al., 2014). The PFTN pigs were presented this total amount of feed in 3 aliquots over the 12 h period (0, 4, and 8 h). No feed refusals where observed.

The challenge experiment was conducted in 8 replicates containing 1 pig from each of the 4 treatment groups. Each HS and TN room's temperature and humidity were continuously recorded every 5 min by

Table 1. Ingredients and formulated dietary nutrients (as-fed basis)

Ingredient, %	Control	ZnAA
Corn	80.56	80.51
Soybean meal, 46.5% CP	15.34	15.35
Soybean oil	1.00	1.00
L-Lysine hydrochloride	0.29	0.29
DL-Methionine	0.40	0.40
L-Threonine	0.08	0.08
Monocalcium phosphate, 21%	0.81	0.81
Limestone	1.00	1.00
Salt	0.50	0.50
Vitamin premix[1]	0.14	0.14
Trace mineral premix[2]	0.05	0.05
Availa zinc 100[3]	–	0.07
Zinc sulfate, monohydrate	0.02	–
Ferrous sulfate, heptahydrate	0.03	0.03
Manganese sulfate, 29.5%	0.02	0.01
Copper sulfate, 25.2% Cu	0.10	0.10
Calcium iodate, 63.5%	0.0002	0.0002
Sodium selenite	0.02	0.02
Calculated composition		
ME, kcal/kg	3,370	3,368
NE, kcal/kg	2,523	2,521
CP, %	14.2	14.2
Total zinc,[4] mg/kg	120	120
Available phosphorus, %	0.23	0.23

[1]Provided the following per kilogram of diet: 7,656 IU vitamin A, 875 IU vitamin D, 62.5 IU vitamin E, 3.75 mg vitamin K, 13.75 mg riboflavin, 70 mg niacin, 33.75 mg pantothenic acid, and 62.5 μg vitamin B_{12}.

[2]Provided the following per kilogram of diet: 121 mg Fe as ferrous sulfate, 121 mg Zn as zinc sulfate, 28.6 mg Mn as manganese sulfate, 12.1 mg Cu as copper sulfate, 0.22 mg I as calcium iodate, and 0.22 mg Se as sodium selenite.

[3]Ingredient profile (Anderson and Abdel-Monem 1997)

[4]The control diet contained 120 mg/kg of $ZnSO_4$; ZnAA = zinc AA complex 60 mg/kg $ZnSO_4$ and 60 mg/kg Availa Zinc100 (Zinpro Corporation, Eden Prairie, MN).

data recorders (Lascar model EL-USB-2-LCD; Lascar Electronics Inc., , Erie, PA). Over the 12-h period, pig thermal status (rectal temperature [**Tr**] and respiratory rate) and FI were recorded every 2 h. Body weights were determined again at the beginning (0 h) and immediately before sacrifice (12 h).

Blood and Tissue Sample Collections

Blood was obtained via jugular venipuncuture immediately before sacrifice. Blood was collected in tubes for K_2EDTA plasma (10 mL; Becton, Dickinson and Company, Franklin Lakes, NJ), blood RNA (Tempus tubes; Life Technologies, Grand Island, NY), serum (10 mL; Becton, Dickinson and Company), or lithium heparin (5 mL; Becton, Dickinson and Company). Harvested blood was centrifuged at 1,300 × *g* for 10 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. In 8 replicates containing 1 pig from each treatment group, pigs were sacrificed after 12 h of HS using barbiturate overdose (Fatal Plus dosed at 1 mL/4.5 kg BW; Vortech Pharmaceuticals, Ltd., Dearborn, MI.).

Intestinal tissues were harvested immediately following euthanasia and included whole sections from both the proximal ileum (approximately 2 m before the ileal-cecal junction) and distal colon (50 cm before the rectum). Fresh sections of whole ileum and colon were flushed of luminal contents, immediately placed into Krebs–Henseleit buffer under constant aeration, and transported to the laboratory for mounting into modified Ussing chambers (Gabler et al., 2007). In addition, tissue samples were snap-frozen in liquid nitrogen and stored at –80°C until later analysis.

Ex Vivo Intestinal Integrity Measures

Ileal and colonic segments from each animal were mounted into modified Ussing chambers (Physiological Instruments, San Diego, CA, and World Precision Instruments, Sarasota, FL) 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 Krebs–Henseleit buffer and tissue was provided with a constant O_2–CO_2 mixture. Individual segments were then voltage clamped (0 mV) and, after 30 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 fluorescein isothiocyanate–labeled dextran (FD4; Sigma-Aldrich) macromolecule permeability as previously described (Pearce et al., 2013b).

Intestinal Morphology Assessment and Zinc Concentrations

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 of intestinal tissues and structures. Villi height, crypt depth, and their ratio was assessed as previously described (Gabler et al., 2007; Pearce et al., 2013c). Frozen ileum and colon segments, flushed of luminal contents, where freeze ground and pooled within tissue and treatment. Tissue zinc concentrations were assayed at the Iowa State University Veterinary Diagnostic Laboratory and expressed in mg/kg.

Blood Analysis

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, total CO_2, partial CO_2, bicarbonate (HCO_3), pH, glucose, blood urea nitrogen, hemoglobin, hematocrit, anion gap, and base excess of the extracellular fluid. Plasma insulin, NEFA, lysozyme activity, alkaline phosphatase activity, lipopolysaccharide binding protein (**LBP**), serum tumor necrosis factor α (**TNF-α**), endotoxin, and haptoglobin were all determined as previously described (Mani et al., 2013; Pearce et al., 2013b,c).

Tissue Protein Expression

Whole cell protein from the ileum and colon was extracted and separated by SDS-PAGE, and semiquantitative protein abundance of heat shock protein 70 (**HSP70**) and hypoxia-inducible factor 1α (**HIF-1α**) was determined as previously described (Pearce et al., 2013b,c). Ileum mucin 2 concentrations were determined using a commercially available ELISA (Porcine MUC 2 ELISA kit; MyBioSource, San Diego, CA) after whole ileum proteins were extracted using phosphate buffered saline (pH 7.2) containing protease inhibitors. The sample protein concentration was determined using bicinchoninic acid assay (Pierce, Rockford, IL) and mucin 2 was expressed as nanograms per milligram of protein.

Ribonucleic Acid Extraction and mRNA Abundance Analysis

Total RNA was isolated from whole ileum and colon mucosal scrapings 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 PCR was performed using a BioMark HD system (Fluidigm Corporation, San Francisco, CA). Complementary DNA from tissues were used for specific target amplification using the TaqMan PreAmp Master Mix (Life Technologies) 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 S1 (see the online version of the article at http://journalofanimalscience.org). 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 quantitative PCR array to select for an endogenous reference gene that was not affected by treatment. The mRNA abundance values for each sample were then normalized to RPL32, which was not affected by the treatments ($P < 0.05$), and a pooled reference sample according to the $2^{\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistical Analysis

All data were statistically analyzed using the MIXED procedure of SAS version 9.2 (SAS Inst. Inc., Cary, NC). Data are reported as least squares means and considered significant if $P \leq 0.05$ and a tendency if $0.05 > P \leq 0.10$. The model included comparison of all 4 treatments as a fixed effect, and the random effect of replicate was included if significant. For hourly measurements (rectal temperature, respiration rate, and FI), each animal's respective parameter was analyzed using repeated measures with an autoregressive covariance structure and time as the repeated effect. The model included treatment, time, and treatment × time interaction. For repeated measures, the individual animal's values at time zero were used as a covariate.

RESULTS

Phenotypic Responses to Heat Stress

There were no differences in Tr between TN-CON and PFTN-CON pigs throughout the 12-h experimental period. Both HS-CON and HS-ZnAA pigs had markedly increased ($P < 0.001$) Tr compared with both TN-CON and PFTN-CON pigs (Table 2; Fig. 1a). Compared with HS-CON pigs, HS-ZnAA pigs had a reduced Tr starting at 4 h and this persisted until 12 h. This decrease in Tr averaged 0.32°C and was most pronounced at 6 h ($P < 0.001$). Both HS-CON and HS-ZnAA pigs had increased (greater than 3-fold) respiration rates compared with both TN-CON and PFTN-CON pigs; however, there were no differences between HS-CON and HS-ZnAA pigs (Table 2; Fig. 1b).

Feed intake was not measured during the 17-d enrichment period and BW were not different as a result of dietary treatment. During the 12-h challenge period, HS-CON and HS-ZnAA pigs had a similar reduction in FI (–0.9 kg/12 h) compared with TN-CON pigs (1.06 kg/12 h; $P < 0.050$; Table 2). By experimental design, the PFTN-CON pigs' FI mirrored that of the HS-CON pigs (Table 2). The TN-CON pigs had a slight BW gain, whereas PFTN-CON, HS-CON, and HS-ZnAA pigs lost BW ($P < 0.001$; Table 1). There were no differences in the amount of BW loss between the HS-CON and HS-ZnAA pigs ($P = 0.450$; Table 2). However, both HS-CON and HS-ZnAA groups lost more weight ($P < 0.001$) than the PFTN-CON pigs.

Intestinal tissue zinc concentrations did not differ due to diet and environmental treatments. Ileum zinc concentrations were 15, 17, 16, and 18 mg/kg for HS-CON, HS-ZnAA, PFTN-CON, and TN-CON, respectively. Colon tissue zinc concentrations were 21, 20, 23, and 19 mg/kg for HS-CON, HS-ZnAA, PFTN-CON, and TN-CON, respectively.

Heat Stress and Pair-Feeding Induced Changes in Intestinal Integrity

Overall, the HS-CON and PFTN-CON pigs experienced greater than a 3-fold increase in ileal FD4 transport compared with the TN-CON pigs ($P < 0.001$; Fig. 2a). The HS-ZnAA pigs had an intermediate FD4 permeability, which was not different from either TN-CON or HS-CON pigs ($P = 0.361$; Fig. 2a). The HS-CON and PFTN-CON pigs also had a 31% decrease in ileal TER compared with the TN-CON group ($P < 0.001$; Fig. 2b). Compared with the HS-CON group, HS-ZnAA pigs had a higher TER (30.2%; $P = 0.052$; Fig. 2b), indicating numerically greater intestinal barrier integrity. Interestingly, 12 h of exposure to HS and pair-feeding to HS FI levels did not alter colonic FD4 permeability and TER ($P > 0.100$; data not shown).

Blood Chemistry and Metabolites

Plasma LBP was decreased 50% ($P = 0.029$; Table 3) in HS-CON pigs compared with TN-CON pigs. However, the HS-ZnAA pigs had LBP plasma concentrations similar to the TN-CON pigs ($P > 0.050$; Table 3). Serum endotoxin was increased in both PFTN-CON and HS-CON pigs (250 and 400%, respectively; $P = 0.017$; Table 3). Intriguingly, HS-

Table 2. Average 12-h core temperatures, respiration rates, feed intake, and BW of pigs reared in thermal neutral (TN; 21°C) or heat stress (HS; 37°C) conditions. Pigs were fed zinc AA complex or control diets ad libitum or pair-fed to HS intake under TN conditions.

	Treatment[1]					
Parameter	TN-CON	PFTN-CON	HS-CON	HS-ZnAA	SEM	*P*-value
Rectal temperature, °C	39.2[a]	39.1[a]	41.8[b]	41.5[c]	0.10	<0.001
Respiration rate, bpm[2]	42.1[a]	41.9[a]	154.3[b]	155.9[b]	2.70	<0.001
Feed intake, kg	1.06[a]	0.18[b]	0.11[b]	0.12[b]	0.08	<0.001
BW change, kg	0.39[a]	–1.87[c]	–4.56[b]	–4.07[b]	0.43	<0.001

[a–c]Different letter superscripts within row differ ($P < 0.05$).

[1]TN-CON = TN conditions with ad libitum intake; PFTN-CON = pair-feeding to their HS-CON counterparts and exposed to TN conditions; HS-CON = heat stress with ad libitum intake; HS-ZnAA = the ZnAA diet ad libitum under heat stress. $n = 8$ pigs/treatment.

[2]bpm = beats per minute.

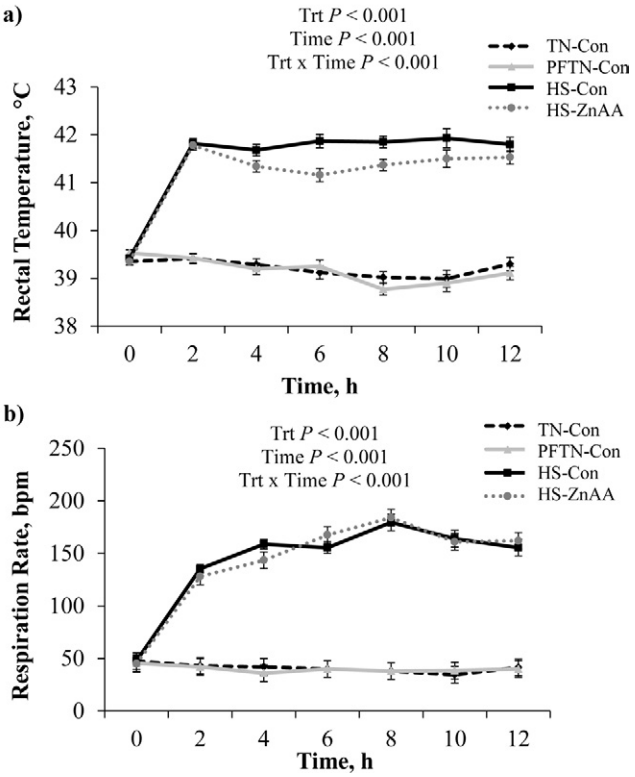

Figure 1. Temporal changes in (a) rectal temperature, and (b) respiration rates in pigs exposed to thermal neutral conditions with ad libitum intake (TN-CON; 21°C), heat stress with ad libitum intake (HS-CON; 37°C), the ZnAA diet ad libitum under heat stress (HS-ZnAA), or pair-feeding to their HS-CON counterparts and exposed to TN conditions (PFTN-CON) for 12 h. bpm = breaths per minute; Trt = treatment

ZnAA pigs had endotoxin concentrations similar to TN-CON pigs. Plasma lysozyme was increased in PFTN-CON and HS-ZnAA pigs compared with both TN-CON and HS-CON pigs ($P < 0.001$; Table 3). Plasma alkaline phosphatase and haptoglobin were unchanged ($P = 0.250$; Table 3) due to environmental temperature or dietary treatment. Serum TNF-α was decreased due to HS, regardless of dietary treatment, compared with both TN-CON and PFTN-CON pigs ($P < 0.001$; Table 3).

Plasma urea nitrogen (**PUN**) was increased ($P < 0.001$) in HS-CON and HS-ZnAA pigs by 1.9- and 1.1-fold compared with TN-CON and PFTN-CON controls (Table 3); however, HS-ZnAA pigs had lower PUN compared with HS-CON pigs ($P < 0.050$). Plasma NEFA were increased 180% ($P < 0.001$; Table 3) in both HS-CON and HS-ZnAA pigs compared with their TN-CON counterparts; however, PFTN-CON pigs did not differ from TN-CON pigs. Plasma insulin was decreased in PFTN-CON, HS-CON, and HS-ZnAA pigs compared with their TN-CON counterparts ($P < 0.050$; Table 3), with HS pigs having the lowest insulin concentrations.

Blood potassium concentration was lower in the HS-CON pigs compared with the TN-CON, PFTN-CON, and HS-ZnAA groups ($P = 0.013$; Supplemental Table S2; see the online version of the article at http://journalofanimalscience.org). There were no differences detected between HS-CON and HS-ZnAA pigs (Supplemental Table S2; see the online version of the article at http://journalofanimalscience.org). Chloride levels were increased (5.7%; $P = 0.018$) in the HS-CON pigs compared with TN-CON, PFTN-CON, and HS-ZnAA pigs. Total CO_2 was lower (10%) in HS-CON, PFTN-CON, and HS-ZnAA pigs compared with TN-CON pigs ($P = 0.03$; Supplemental Table S2; see the online version of the article at http://journalofanimalscience.org). Heat stress increased blood pH (2%) in both HS-CON and HS-ZnAA groups compared with their TN-CON and PFTN-CON counterparts ($P < 0.001$; Supplemental Table S2; see the online version of the article at http://journalofanimalscience.org). Partial CO2 was decreased by 36% in both HS groups. Bicarbonate was decreased 11% in HS-CON, PFTN-CON, and HS-ZnAA pigs compared with their TN-CON counterparts ($P = 0.040$; Supplemental Table S2; see the online version of the article at http://journalofanimalscience.org). There were no treatment differences in glucose concentrations, hematocrit, sodium, base excess, anion gap, or hemoglobin ($P > 0.050$; Supplemental Table S2; see the online version of the article at http://journalofanimalscience.org).

Ileum Histology, Mucin, and Stress Response

Twelve hours of HS tended to reduce ileal villi height compared with the TN, PFTN, and ZnAA treatments (342, 428, 421, and 393 μm, respectively; $P = 0.058$). There were no differences in crypt depth among the 4 treatments ($P = 0.996$; data not shown). However,

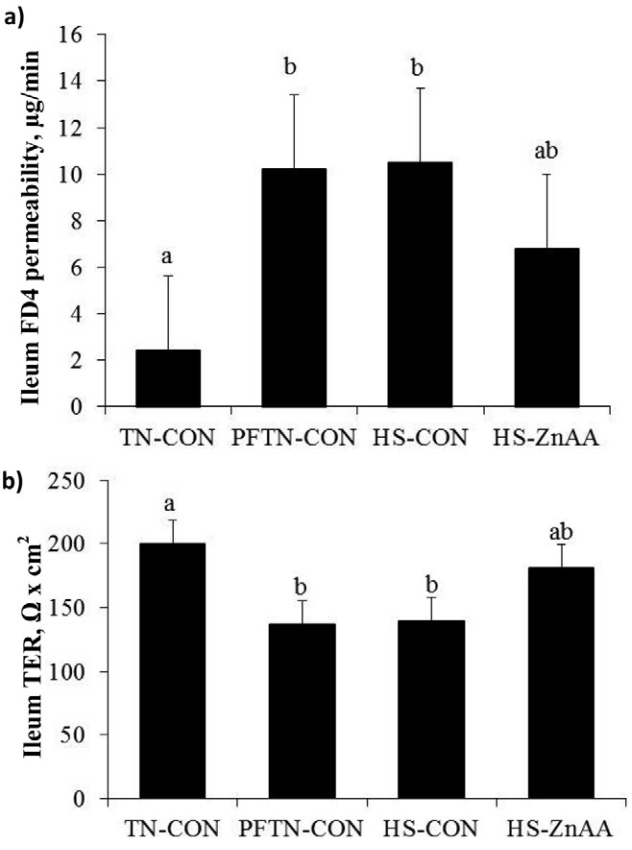

Figure 2. Changes in (a) ileal 4.4 kDa fluorescein isothiocyanate–labeled dextran (FD4) permeability and (b) ileal transepithelial electrical resistance (TER) in pigs exposed to thermal neutral conditions with ad libitum intake (TN-CON; 21°C), heat stress with ad libitum intake (HS-CON; 37°C), pair-feeding to their HS-CON counterparts and exposed to TN conditions (PFTN-CON), or the ZnAA diet ad libitum under heat stress (HS-ZnAA) on feed intake. $^{a,b}P < 0.05$.

the ratio of villi height to crypt depth was reduced due to HS-CON versus TN-CON and PFTN-CON but not HS-ZnAA ($P = 0.048$; 1.2, 1.7, 1.6, and 1.5, respectively). Additionally, the HS-CON ileal sections had a marked increase in autolysis compared with the HS-ZnAA, PFTN-CON, and TN-CON sections (Fig. 3).

To locally assess the hypoxia and stress response in ileum and colon tissue, HSP70 and HIF-1α protein expression were examined (Fig. 4). In the ileum (Fig. 4a), HS-CON and HS-ZnAA HSP70 expression increased compared with the TN-CON and PFTN-CON HSP70 expression ($P < 0.050$). Although numerically increased due to HS, ileum HIF-1α protein expression was not different between treatments ($P = 0.152$). Colonic (Fig. 4b) HIF-1α protein expression did not differ ($P = 0.815$), but HS-CON colonic tissue had a 200% increase in HSP70 protein expression compared with the HS-ZnAA and TN-CON colonic tissue ($P < 0.050$). Furthermore, compared with the control pigs, ileal mucin 2 protein expression was also increased 2-fold due to HS and PFTN treatments ($P < 0.050$; Fig. 5).

Intestine mRNA Abundance

Changes in ileum and colonic tight junction and in inflammation and integrity gene abundances after 12 h of HS or PFTN are shown in Supplemental Tables S3 and S4 (see the online version of the article at http://journalofanimalscience.org), respectively. In the ileum, only 1 gene (integrin β-1) was found to be altered ($P < 0.050$; Supplemental Table S3; see the online version of the article at http://journalofanimalscience.org) and was increased in HS-CON pigs compared with both PFTN-CON and HS-ZnAA pigs. There was also a tendency for IL1-β to be decreased in HS-ZnAA pigs compared with TN-CON pigs. No other significant changes in ileum gene abundances were reported.

In the colon, claudin-4 abundance was significantly increased only due to PFTN ($P = 0.003$). However, occludin gene abundance was decreased ($P = 0.026$; Supplemental Table S4; see the online version of the article at http://journalofanimalscience.org) in HS-CON pigs compared with TN-CON pigs whereas HS-ZnAA pigs were not different from either treatment. Intestinal alkaline phosphatase gene abundance was increased (133%; $P = 0.045$; Supplemental Table S4; see the online version of the article at http://journalofanimalscience.org) in HS-ZnAA pigs compared with both HS-CON and TN-CON pigs. Colon mucin-2 mRNA abundance as well as *TLR-4* abundance was significantly elevated in HS-ZnAA pigs ($P = 0.031$; Supplemental Table S4; see the online version of the article at http://journalofanimalscience.org). Mast cell tryptase was decreased due to HS but maintained by dietary zinc treatment ($P = 0.019$; Supplemental Table S4; see the online version of the article at http://journalofanimalscience.org). Heme oxygenase-1 was elevated in PFTN-CON and HS-ZnAA pigs but HS-CON pigs were not different from their TN-CON counterparts (Supplemental Table S4; see the online version of the article at http://journalofanimalscience.org).

DISCUSSION

Zinc promotes normal intestinal barrier function and the regeneration of damaged gut epithelium (Alam et al., 1994). Zinc has also been shown to effectively prevent or ameliorate the loss of barrier function under malnutrition (Rodriguez et al., 1996), alcoholic liver disease (Zhong et al., 2010), chronic inflammatory bowel or Crohn's diseases (Sturniolo et al., 2001), and infectious diarrhea (Alam et al., 1994). Therefore, we were interested in further examining the ability of organic and inorganic dietary zinc sources to attenuate changes in intestinal integrity and function on pigs subjected to HS. Previous research has shown that increasing levels of zinc have been beneficial; however, we wanted to deter-

Table 3. Blood chemistry and ion and metabolite concentrations in pigs reared under heat stress (HS; 37°C), dietary zinc, and pair-feeding thermal neutral conditions (21°C) for 12 h

	Treatment[1]					
Parameter	TN-CON	PFTN-CON	HS-CON	HS-ZnAA	SEM	*P*-value
PUN,[2] mg/dL	32.3[a]	36.5[a]	98.3[c]	72.4[b]	8.11	<0.001
Glucose, mg/dL	99.2	98.0	93.4	97.0	4.08	0.678
Plasma NEFA, mmol/L	0.09[a]	0.12[a]	0.25[b]	0.25[b]	0.029	<0.001
Insulin, ng/dL	0.18[c]	0.10[b]	0.04[a]	0.08[ab]	0.020	<0.001
Alkaline phosphatase, units/L	55.3	45.2	53.6	42.4	5.20	0.250
Plasma lysozyme, units/mL	173.8[a]	194.8[b]	166.9[a]	188.6[b]	4.26	<0.001
TNF-α,[3] pg/mL	73.5[a]	85.1[a]	48.3[b]	52.0[b]	6.23	<0.001
Haptoglobin, mg/mL	101.4	137.5	70.4	115.9	23.30	0.256
Plasma LBP,[4] μg/mL	14.12[b]	10.47[ab]	7.22[a]	15.46[b]	2.598	0.029
Serum endotoxin, AU[5]	1.00	3.51	5.10	2.31	0.868	0.057

[a,b]Within a row, different superscripts differ ($P < 0.05$).

[1]TN-CON = thermal neutral conditions with ad libitum intake; PFTN-CON = pair-feeding to their HS-CON counterparts and exposed to TN conditions; HS-CON = heat stress with ad libitum intake; HS-ZnAA = the ZnAA diet ad libitum under heat stress. $n = 8$ pigs/treatment.

[2]PUN = plasma urea nitrogen.

[3]TNF-α = tumor necrosis factor **α**.

[4]LBP = lipopolysaccharide binding protein.

[5]AU = Arbitrary units.

mine whether supplementing with an adequate level of zinc AA complex would be beneficial as well. The amount of zinc we chose for both diets (120 mg/kg) is higher than what the NRC (1998) recommended as adequate; however, these levels are not toxic and are routinely used in commercial swine diets.

These pigs were acutely exposed (12 h) to a severe heat load (constant 37°C and 40% humidity). These HS conditions were chosen to mimic the physiological effects of severe HS. Heat-stressed pigs have been shown to increase average daily water intake by 34% compared with TN control pigs (Song et al., 2011). Although we did not measure water intake in the current study, we believe our HS pigs were well hydrated as assessed by blood hematocrit values, which were not different from the TN control pigs.

By design, our pigs experienced a heat load far above their thermal comfort zone, and this resulted in a marked augmentation in all body thermal indices measured (Tr and respiration rate). The average difference in Tr between the TN and the HS pigs was 2.6°C. Interestingly, HS-ZnAA pigs had a lower body temperature (0.3°C) compared with HS-CON pigs from 4 h onward. These data are different from our previous work, in which we did not observe any improvement in core temperatures of HS-ZnAA pigs that were fed much higher concentrations of dietary Zn amounts and experienced HS for a longer period (Sanz Fernandez et al., 2014). The reduction in Tr is difficult to interpret, as HS-CON and HS-ZnAA pigs had similar FI and change in BW, 2 key variables associated with basal heat production (Renaudeau et al., 2013).

The deleterious effects of HS are mediated, at least in part, by its effects on gastrointestinal health and function (Eshel et al., 2001). 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 lead to hypoxia, oxidative and nitrosative stress in the enterocyte (Lambert, 2004; Pearce et al., 2013a,b,c). As a result, cell and mucosal membranes and tight junctions can be damaged, leading to an increase in intestinal permeability (Lambert et al., 2002). We report herein an increased passage of high molecular weight substances (FD4) and circulating endotoxin due to HS. The increased autolysis observed in our histology and changes in markers of intestinal integrity (TER) also strongly support this phenotypic response. However, organic zinc supplementation reduced the severity of this HS response compared with the inorganic zinc–HS treatment. Moreover, reduced FI (PFTN-CON) appears to be a major contributing factor to this increased intestinal dysfunction. We (Pearce et al., 2013c) and others (Ferraris and Carey, 2000) have previously reported that restricted nutrient intake can lead to alterations in intestinal function, transport, and morphology and this may increase the risk of developing bacterial sepsis. Therefore, the effects observed during HS may be partially explained or exacerbated by the dramatic reduction in FI.

The mode of action of organic zinc versus inorganic zinc in regulating intestinal function and integrity may be related to its improved bioavailability (Glover et al., 2003). Overall, dietary zinc has been reported to act mainly at the tight junction level. For example, in vitro

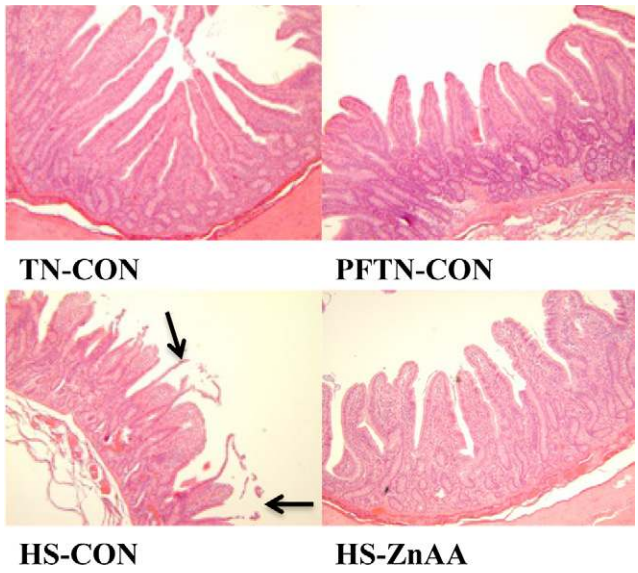

Figure 3. Heat stress induced alterations in ileum morphology. Hemotoxylin and eosin sections from pigs exposed to thermal neutral conditions with ad libitum intake (TN-CON; 21°C), heat stress with ad libitum intake (HS-CON; 37°C), pair-feeding to their HS-CON counterparts and exposed to TN conditions (PFTN-CON), or the ZnAA diet ad libitum under heat stress (HS-ZnAA) for 12 h. Arrows indicate areas of pronounced autolysis.

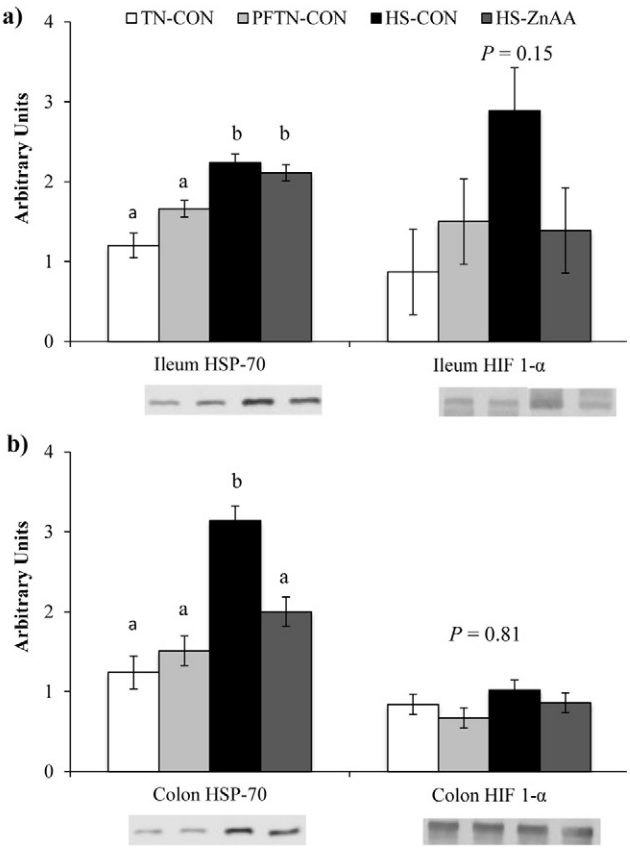

Figure 4. Changes in pig (a) ileum and (b) colon heat shock protein 70 (HSP70) and hypoxia-inducible factor 1α (HIF-1α) protein expression after 12 h of thermal neutral conditions with ad libitum intake (TN-CON; 21°C), heat stress with ad libitum intake (HS-CON; 37°C), pair-feeding to their HS-CON counterparts and exposed to TN conditions (PFTN-CON), or the ZnAA diet ad libitum under heat stress (HS-ZnAA). a,b$P < 0.05$. n = 8/treatment, and all semiquantitative data was adjusted relative to GAPDH protein expression.

studies indicate that Caco-2 cells grown in zinc-depleted media have a decreased amount of tight junctions and a compromised cytoskeleton (Finamore et al., 2008). Similarly, zinc deficiency reduces ileal tight junction protein expression in an alcohol-induced intestinal barrier dysfunction model (Zhong et al., 2010). However, zinc supplementation prevented the opening of these junction complexes in a rat colitis model (Sturniolo et al., 2001). Zinc oxide supplementation has been shown to reduce intestinal permeability in weaning piglets while increasing the amount and expression of tight junction proteins (Zhang and Guo, 2009). In a previous study, we have shown that increasing levels of zinc AA complex (220 and 320 mg/kg) increased gastrointestinal tract integrity compared with control animals, which were fed 120 mg/kg zinc sulfate (Sanz Fernandez et al., 2014). Interestingly, presented herein, we reported limited to no changes in the abundance of many intestinal tight junction and integrity related genes due to HS. This may indicate that at the gene transcription level, HS is not having a large impact at 12 h. Additionally, many tight junction and barrier function proteins undergo posttranslational modification. Therefore, gene abundance may not fully explain the observed phenotype or protein functionality. Another explanation for the lack in gene abundance differences may be due to the increased autolysis and sloughing of the intestinal epithelial layer due to HS. Morphological analysis of the ileum showed shortening of villi and linear laminar separation as well as entire layers of cells sloughing. Although shortening of villi also occurred in PFTN-CON and HS-ZnAA animals, the damage was not as severe when compared with the HS-CON pigs, indicating another possible mechanism by which dietary zinc may improve intestinal health under stress.

Epithelial mucins and the mucus layer produced by goblet cells in the intestinal epithelium serve as a critical component in the barrier defense against pathogen colonization and permeability. In the intestinal tract, mucin 2 is the predominant secretory mucin (Van der Sluis et al., 2006). In the present study, we report that HS and feed restriction increased mucin 2 protein expression in the ileum of pigs compared with TN-reared pigs. Our HS data agrees with Ashraf et al. (2013), who reported that cyclic HS increased acidic and mixed mucin contents in broiler duodenum and ileum segments compared with TN-reared birds. However, Liu et al. (2014b) recently reported that the jejunum mucin gene abundance was not altered in nursery pigs supplemented with increasing amounts of zinc oxide (50–2,400 mg/kg). Irrespectively, others have reported a strong link between mucin 2 and intestinal barrier function (Zarepour et al., 2013). This study showed mucin 2 knockout mice had impaired intestinal alkaline phosphatase and endotoxin detoxifi-

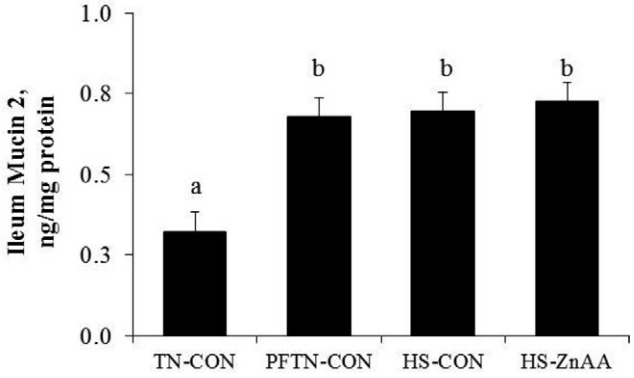

Figure 5. Twelve hours of either heat stress and feed restriction increased ileum Mucin 2 protein expression; $n = 8$/treatment; $^{a,b}P < 0.05$. TN-CON = thermal neutral conditions with ad libitum intake; HS-CON = heat stress with ad libitum intake; PFTN-CON = pair-feeding to their HS-CON counterparts and exposed to TN conditions; HS-ZnAA = the ZnAA diet ad libitum under heat stress.

cation. Based on our findings, we speculate that the increase in mucin production in response to HS is to help maintain the protective intestinal barrier and its structural integrity. Mechanistically, mucin secretion increases with acute stress as a result of increased corticotropin-releasing factor (Castagliuolo et al., 1996). Corticotropin-releasing factor is a central and peripheral stress mediator and has also been directly linked to augmented intestinal dysfunction associated with weaning stress in pigs (Smith et al., 2010). Although high dietary zinc has also been shown to increase the amount intestinal mucin in swine (Liu et al., 2014a), other studies (Hedemann et al., 2006) and our data herein suggest that this is independent of zinc source.

Circulating concentrations of endotoxin were elevated in HS and PFTN pigs. This is consistent with our previous work (Pearce et al., 2013b,c) and is related to the reduction in intestinal integrity and/or reduced intestine and liver capacity to detoxify endotoxin molecules (Mani et al., 2012). In pigs, weaning stress (Lackeyram et al., 2010) and fasting (Lalles and David, 2011) have been shown to reduce intestinal endotoxin detoxifying capacity. However, we observed few differences in key gene abundances associated with endotoxin detoxification and clearance (*AOAH*, *IAP*, and *CYTC*).

Heat stress evokes a rapid heat shock response involving the activation of heat shock proteins (Pearce et al., 2014). Heat shock proteins are a large family of stress proteins that are highly conserved across species and generally serve as molecular chaperones or stabilizers of protein denaturation and in cytoprotection. Their activity is prevalent within a few hours of the onset of severe stress and lasts for a few days (Horowitz, 2002). Expression of these proteins is mediated by activation of heat shock factor 1 (HSF1), which binds to heat shock elements and initiates transcription of several heat shock genes (Singh and Hasday, 2013). One of the major inducible heat shock protein family members is HSP70–72 kDa, which aids in protein folding, ubiquination, and stabilization and providing protection during times of cellular stress (Petrof et al., 2004). In the present study, we have reported the induction of HSP70 protein expression in the ileum and colon of HS pigs. Specific to thermal biology, HSP70 is rapidly increased 2 to 4 h after heat exposure (Dokladny et al., 2006). With that, zinc has been shown to increase the expression of HSP-70/72 (Odashima et al., 2002; Lodemann et al., 2013). However, proteomic analysis of zinc oxide–treated pig hepatic tissue have reported 150 mg/kg zinc to downregulate HSP70 and this fits with our current results.

Often accompanied with the heat shock and oxidative stress response is hypoxia. Hypoxia is regulated by a complex system of oxygen-sensing mechanisms. Hypoxia-inducible factors are transcriptional regulators that play a role in oxygen homeostasis. Similar to our previous studies (Pearce et al., 2013b,c), in the current study, HS numerically induced HIF-1α protein in the ileum but not the colon of pigs. This augmentation of HIF-1α was attenuated by ZnAA treatment. Tissue differences in the expression of HIF-1α are most likely explained by differences in blood flow and reduced oxygen delivery to the intestinal tract (Yoshitake et al., 1998). From a dietary standpoint, we speculate that ZnAA may be better at inducing proteasomal degradation of HIF-1α, as zinc treatment downregulates HIF-1α in human tumor cells by this mechanism (Nardinocchi et al., 2010).

In many species, including rodents (Helwig and Leon, 2011; Biedenkapp and Leon, 2013; Welc et al., 2013), cattle (Carroll et al., 2013), and humans (Leon and Helwig, 2010), HS causes both local and systemic inflammation during and after exposure. Part of this inflammatory response may be the result of endotoxemia, due to increased intestinal permeability (Lambert, 2009; Pearce et al., 2013c). The poultry literature has reported that supplemental dietary Zn has an anti-inflammatory effect during HS (Sahin et al., 2009). However, swine appear to have a differing proinflammatory response during HS. Herein and previously (Pearce et al., 2013c; Montilla et al., 2014), we have reported growing pigs exposed to HS conditions to have a reduced blood and tissue proinflammatory cytokine response yet still mount an acute phase response. However, one explanation for the reduced inflammation could be the augmentation of the heat shock protein response. Inducible heat shock proteins, a product of heat shock, have been shown to inhibit nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) pathway activation (Chen et al., 2006). Additionally, heat shock pretreatment has been used to attenuate endotoxin-induced sepsis–associated encephalopathy (Lin et al., 2010). Altogether, these

highlight a key anti-inflammatory signaling role of heat shock proteins.

Heat stress markedly alters protein metabolism in a number of animal species independently 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-methylhystidine, a better marker of muscle catabolism (Yoshizawa et al., 1997; Pearce et al., 2013a). Although HS-ZnAA pigs had an increased blood urea nitrogen compared with TN-CON pigs, it was lower than our HS-CON group.

In conclusion, despite both diets having similar dietary zinc concentrations and only differing in zinc source, dietary zinc AA complex minerals improved several aspects of intestinal integrity in pigs subjected to HS conditions. These include increasing epithelial resistance, maintaining epithelial cell morphology, decreasing circulating endotoxin, and increased acute phase response (LBP and lysozyme). Additionally, zinc AA complex resulted in improvements in blood markers of muscle catabolism as indicated by decreased PUN. Caloric restriction also may explain a large majority of detrimental effects on intestinal integrity. Taken together, zinc AA complex may prove a valuable dietary mitigation strategy for HS, but it is important to note that the current study implements zinc as a prevention with prior enrichment, as opposed to a medical treatment.

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