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Non ruminant nutrition

Effect of DL-methionine supplementation above requirement on performance; intestinal morphology, antioxidant activity, and gene expression; and serum concentration of amino acids in heat stressed pigs

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Abstract

The intestinal morphology and function can be compromised in pigs exposed to heat stress (**HS**), partly due to increased production of reactive-oxygen species. Because methionine (**Met**) functions as intracellular antioxidant, the requirement of Met may be increased in HS-pigs. The effect of dietary supplementation with DL-Met above requirement on performance, small intestine morphology, antioxidant enzymes activity, amino acid transporters expression, and serum concentration (**SC**) of free AA in HS-pigs was evaluated. A basal wheat–soybean meal diet was formulated to meet 100% Met requirement with the other indispensable AA exceeding at least 20% their requirement. Sixty individually housed pigs (23.0 ± 2.4 kg BW, 12 pigs per treatment) were randomly assigned to five treatments: TN100, thermal-neutral (22.7 °C) housed pigs fed the basal diet; HS100, HS120, HS140, HS160; HS-pigs (29.6 °C to 39.4 °C) fed the basal diet supplemented with DL-Met to contain 0%, 20%, 40%, and 60% DL-Met above the requirement, respectively. Pigs had free access to feed and water during the 21-d trial. Blood samples were collected on day 18 to analyze the absorptive AA-SC. The effect of ambient temperature (HS100 vs. TN100), as well as the linear and quadratic effects of increasing Met levels in the diets for HS-pigs were analyzed. The HS100 pigs gained less weight than TN100 and HS120 pigs ($P < 0.01$); gain:feed was also higher in HS120 pigs than in HS100 pigs ($P \leq 0.05$). Feed intake of TN100 pigs was higher than that of HS-pigs fed the DL-Met supplemented diets ($P < 0.05$). Villi height reduced in pigs HS, but Met supplementation quadratically increased it ($P < 0.05$). Superoxide dismutase and catalase activities, reduced glutathione concentration, and relative expression of B^0AT2 in ileum decreased ($P < 0.05$), but glutathione peroxidase activity increased in HS-pigs. DL-Met supplementation linearly affected catalase and glutathione peroxidase activities, as well as the relative expression of $b^{0,+}AT$ in jejunum ($P < 0.05$) of HS-pigs. The SC of Ile, Leu, Lys, Phe, and Val were higher in HS100 pigs than in TN100 pigs ($P < 0.05$). Graded levels of supplemental DL-Met in diets for HS-pigs linearly decreased SC of Ile, Leu, and Val ($P < 0.05$), tended to decrease His, Lys, and Thr ($P < 0.10$), and increased Met ($P < 0.01$). In conclusion, HS had negative effect on weight gain and intestinal morpho-physiology; however, it was ameliorated by adding 20% Met above the requirement in diets for growing pigs.

Lay Summary

The exposure of pigs to ambient temperature above their comfort zone affects several functions of the small intestine, especially those related with digestion of feed and absorption of nutrients, which in turn reduces the availability of nutrients for growth. Amino acids such as methionine are involved in multiple functions of intestinal cells. Thus, methionine supplementation may help pigs to overcome the negative impact of their exposure to high ambient temperature. Indeed, methionine supplementation to the diet increased growth rate and feed efficiency of pigs housed under heat stress, which was presumably associated with an improvement in the utilization of the absorbed amino acids.

Key words: heat stress, methionine, pigs, serum amino acids

Abbreviations: AA, amino acids; AT, ambient temperature; BT, body temperature; BW, body weight; CD, crypt depth; CP, crude protein; DNA, deoxyribonucleic acid; HS, heat stress; mRNA, messenger RNA; PCR, polymerized chain reaction; qPCR, quantitative PCR; RH, relative humidity; RNA, ribonucleic acid; RPL4, ribosomal protein L4; SC, serum concentration; SID, standardized ileal digestibility; VFI, voluntary feed intake; VH, villi height

Introduction

The exposure of pigs to high ambient temperature (**AT**) provokes heat stress (**HS**), which is characterized by increased body temperature (**BT**; Pearce et al., 2014). In response, HS pigs redirect blood flow to the periphery (Wilson and Crandall, 2011) and reduce feed intake to maintain normal BT. Postprandial BT increments may intensify the impact of HS (Morales et al., 2018). Both blood flow redirection and decreased feed intake, however, reduce the supply of nutrients to the small intestine due to decreased amount of blood reaching internal organs (Ogoh et al., 2013) causing intestinal damage (Pearce et al., 2013) that may affect nutrient absorption. Also, HS cells increase production of reactive-oxygen species (**ROS**; Kikusato and Toyomizu, 2013) that might

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additionally damage the intestinal epithelia. Hence, reduced concentration of free AA in blood serum (**SC**), which is an indicator of AA availability for growth (Yen et al., 2004) may be expected in HS pigs.

Methionine (**Met**) is indispensable for the synthesis of all tissue proteins (Brosnan and Brosnan, 2007) and is the third limiting AA in cereal–soybean meal diets for pigs (NRC, 2012). Moreover, Met functions as an antioxidant either in free form or as protein-bound Met. Residues of Met in cellular antioxidant proteins are highly susceptible to oxidation by ROS, but the oxidized Met may be reduced by a sulfoxide reductase (Moskovitz et al., 2001). Proteins of the respiratory chain (Complex I), where most of ROS production occurs, are abundant in Met (Schindeldecker and Moosmann, 2015). Also, Met is precursor for the synthesis of glutathione (Bauchart-Thevret et al., 2009), important component of the cellular antioxidant system. As such, HS may increase the demand for Met, which may become limiting for growth of HS pigs. We hypothesized that supplementing diets with increased levels of DL-Met may correct its limitation, and improve the Met availability and the utilization of AA in HS pigs. In the present study, the effect of dietary DL-Met supplementation on growth performance, gut morphology, antioxidant activity, and concentration of free AA in serum as well as the expression of genes coding for selected AA transporters in the small intestine of pigs exposed to HS was evaluated.

Materials and Methods

Animal, housing, and dietary treatments

An experiment was conducted with 60 crossbred (Landrace × Hampshire × Duroc) pigs in the Northwestern part of México during the warmest season of the year (July and August). The pigs (23.0 ± 2.4 kg initial body weight) were individually housed in 1.2 × 1.2 m^2 pens inside either a temperature-controlled room (AT, 22 ± 2 °C) or a room without temperature control with the typical AT fluctuations. The pens had elevated iron-mesh floor, and were equipped with a stainless-steel self-feeder and a nipple water drinker to allow ad libitum consumption of feed and water. In addition, four pigs were surgically implanted with a thermometer (Thermotracker BT; iButtonLink LLC, Whitewater, WI, USA) through a cannulae into the lumen of terminal ileum to register the BT at 5-min intervals. The surgery was performed following humanitarian procedures outlined by Sauer et al. (1983) in pigs used previously in a digestion trial hence they were fully recovered from surgery. The respiratory frequency (breaths per min) was measured as the number of abdomen expansions per min in 8 pigs per treatment on days 16 and 18 of the trial at both 0700 and 1700 hours. The AT and relative humidity inside each room were recorded with the aid of a hygrothermograph (Thermotracker HIGRO; iButtonLink LLC, Whitewater, WI, USA) set to record those values every 15 min during the whole study. The pigs used in the present experiment were cared for in accordance with the guidelines established in the Official Mexican Regulations on Animal Care (NOM-062-Z00-1999, 2001).

The pigs were randomly assigned to five treatment groups (Table 1) as follows: 1—pigs housed under TN conditions and fed a basal diet formulated to contain 100% the NRC (2012) requirement of standardized ileal digestible (SID) Met (**TN100**); 2 to 5—pigs housed under HS conditions fed the basal diet (**HS100**) or this diet plus 0.06%, 0.12%, or 0.18% crystalline DL-Met to supply 20% (**HS120**), 40% (**HS140**), and 60% (**HS160**) more Met, respectively, than the basal diet. The DL-Met supplemental levels were chosen to overcome the reduced Met intake because of the reduction in the voluntary feed intake of HS pigs. In addition, 0.06%, 0.12%, and 0.18% free L-Lys.HCl was also supplemented to the HS120, HS140, and HS160 diets, respectively, to match the DL-Met increments. The basal diet was formulated with wheat and soybean meal, as well as free Lys and Thr to contain, with the exception of Met, at least 125% the NRC (2012) SID AA requirements for pigs in the BW range of 25 to 50 kg. The analyzed AA content (Table 2) and the published SID coefficients in wheat and SBM (Stein et al., 2001) were used in the formulation of all diets, and free AA were considered 100% SID. All diets were supplemented with a mineral–vitamin premix to meet or exceed their requirements for growing pigs (NRC, 2012), and contained 10.0 MJ NE/kg. The feed and water were available all the time during the 21-d trial. Pigs were weighed on a weekly basis to calculate the average daily gain (ADG); feed intake (ADFI) and gain:feed (G:F) ratio were registered with the same frequency. Daily intake of AA (g/d) during week 3 (when blood samples were collected for analysis of the SC of free AA) was calculated based on daily feed intake and the analyzed content of each AA in the diets. Within the experimental period, all ileal cannulated pigs were housed inside the HS room to record BT during 5 d, then they were moved to the TN room to record BT again also during 5 d. These pigs were fed the control diet.

Collection of blood and tissue samples

Six pigs from each treatment were slaughtered by electrical stunning and exsanguination at the end of the trial (day 21). The corpses were eviscerated and the following samples were collected: 1) mucosa scratched from duodenum, jejunum, and ileum into 2-mL Eppendorf microtubes and immediately stored in liquid nitrogen for later gene expression analyses and 2) segments of 5 cm from duodenum, jejunum, and ileum stored in 10% formyl buffer for later histology characterization based on the procedure described by Moeser et al. (2012). Blood samples were also collected during the exsanguination of the animals for determining the SC of AA and the activity of antioxidant enzymes.

Chemical and histomorphological analyses

Wheat, soybean meal, and the diets were analyzed for AA content (Method 999.13; AOAC, 2005) after acid hydrolysis with 6 N HCl for 24 h at 110 °C (method 982.30E; AOAC, 2006). Freeze–dried serum samples were also analyzed for free AA and AA metabolites at the chemical lab of University of Missouri-Columbia, USA. The free AA were determined after dissolving the freeze–dried serum samples and carrying out protein precipitation with sulfosalicylic acid and centrifugation. The analyses of AA were performed by ion-exchange chromatography with postcolumn ninhydrin derivatization, and the use of a fluorescence detector. Amino acids were oxidized with performic acid, which was neutralized with Na metabisulfite. The AA were quantified with the internal standard by measuring the absorption of reaction products with ninhydrin at 570 nm.

Segments (5 cm) of duodenum, jejunum, and ileum were fixed in 10% neutral-buffered formaldehyde for paraffin embedding. These formalin-fixed samples were cut and further stained with hematoxylin and eosin (Driscoll and Ryan,

Table 1. Ingredient composition of the experimental diets (exp 1)[1]

Ingredient	Met100	Met120	Met140	Met160
Wheat	85.20	85.20	85.20	85.20
Soybean meal, 48% CP	11.00	11.00	11.00	11.00
L-Lys • HCl	0.55	0.6	0.65	0.70
L-Thr	0.17	0.17	0.17	0.17
Powder DL-Met	0.06	0.12	0.18	0.24
Corn starch	0.32	0.21	0.11	-
Calcium carbonate	1.25	1.25	1.25	1.25
Dicalcium phosphate	0.80	0.80	0.80	0.80
Iodized salt	0.35	0.35	0.35	0.35
Vitamin and mineral premix[2]	0.40	0.40	0.40	0.40
Calculated composition				
NE, MJ/kg	10.02	10.03	10.03	10.04
SID Arg	0.84	0.84	0.84	0.84
SID His	0.37	0.37	0.37	0.37
SID Ile	0.56	0.56	0.56	0.56
SID Leu	1.07	1.07	1.07	1.07
SID Lys	0.99	1.05	1.11	1.17
SID Met	0.28	0.34	0.40	0.46
SID Met + Cys	0.57	0.63	0.69	0.75
SID Phe	0.72	0.72	0.72	0.72
SID Thr	0.61	0.61	0.61	0.61
SID Trp	0.18	0.18	0.18	0.18
SID Val	0.65	0.65	0.65	0.65

[1]Diets: Met100, Control wheat–SBM diet supplying 100% NRC (2012). Met requirement; Met120, Met140, and Met160 contained 20%, 40%, and 60% more Met than the control diet.
[2]Supplied per kg of diet: Vitamin A, 4,800 IU; vitamin D_3, 800 IU; vitamin E, 4.8 IU; vitamin K_3, 1.6 mg; riboflavin, 4 mg; D-pantothenic acid, 7.2 mg; niacin, 16 mg; vitamin B_{12}, 12.8 mg; Zn, 64 mg; Fe, 64 mg; Cu, 4 mg; Mn, 4 mg; I, 0.36 mg; Se, 0.13 mg. The premix was supplied by Nutrionix, S.A., Hermosillo, México.

Table 2. Analyzed amino acid composition of the experimental diets

Diets				
	Met 100	Met120	Met140	Met160
Arg	1.05	1.08	1.07	1.10
His	0.43	0.44	0.43	0.44
Ile	0.71	0.72	0.72	0.73
Leu	1.25	1.27	1.26	1.28
Lys	1.30	1.36	1.39	1.48
Met	0.32	0.36	0.46	0.54
Phe	0.85	0.87	0.86	0.87
Thr	0.84	0.83	0.86	0.90
Trp	0.27	0.27	0.27	0.28
Val	0.88	0.89	0.89	0.91

1978), and the mucosal structure was observed into an optic microscopy (HBO50 Primo Star, Zeiss, Mexico) using 40× magnification. Microphotographs were obtained by a photographic camera (Canon, Tokyo, Japan). Villus height (**VH**) and crypt depth (**CD**) of at least 10 well-oriented villi were measured and analyzed using the software Image J2 (Rueden et al., 2017).

The activities of the antioxidant enzymes superoxide dismutase (**SOD**), catalase (**CAT**), and glutathione peroxidase (**GPX**), and the concentration of reduced glutathione (**GSH**) were analyzed in serum using assay kits (Cayman Chemical Company, Ann Arbor, Michigan 48108, USA). The activities of SOD (assay kit #706003), CAT (assay kit #707002), and GPX (assay kit #703102), and the concentration of GSH were measured following the manufacturer's protocols. The activity of SOD was analyzed using a microplate reader (BioTek Instruments, Inc., USA) at 450 nm, CAT and GPX were analyzed at 340 nm, whereas GSH was analyzed at 412 nm.

RNA extraction and reverse transcription

Total RNA was extracted from the mucosa samples using Trizol reagent (Invitrogen, Corp., Carlsbad, CA, USA), as reported by Méndez et al. (2011). Purified RNA was then eluted with 30 µL of RNase-free water and stored at −82 °C. The concentration of total RNA was determined spectrophotometrically at 260 nm (Helios β, Thermo Electron Co., Rochester, NY, USA), and purity of RNA was assessed by using the A260/A280 ratio, which ranged from 1.8 to 2.0 (Sambrook and Russell, 2001). The integrity of total RNA was evaluated by gel electrophoresis on 1% agarose gels. All RNA samples had good quality with a 28S:18S rRNA ratio around 2.0:1 (Sambrook and Russell, 2001). Approximately 2 µg of total RNA were treated with 1 U of DNase I (1 U/µL; Invitrogen) and the reverse transcription was performed using random hexamers as previously described by (García-Villalobos et al., 2012). The complementary DNA samples were spectrophotometrically quantified and diluted into a final concentration of 50 ng/µL.

Real-time PCR

Specific primers for the AA transporters $b^{0,+}$, y^+L, and B^0 mRNA, and ribosomal protein L4 (RPL4) gene were designed according to their published sequences at the Genbank (Table 3). RPL4 gene was used as an endogenous control to normalize variations in mRNA (Park et al., 2015). Endpoint polymerized chain reaction (PCR) was carried out to standardize the amplification conditions for each pair of primers before starting. The specificity of the PCR products related to its mRNA was confirmed using the PureLink PCR Purification kit (Invitrogen) and sequenced at the Genewiz facility (South Plainfield, NJ, USA). Sequencing results revealed that the products for $b^{0,+}$, y^+L, and B^0, and RPL4 mRNA had 100% homology with their corresponding expected sequences acquired from the virtual template sequences reported in the GenBank.

Expression of mRNA coding for $b^{0,+}$ and B^0 was analyzed by quantitative PCR (qPCR) assays, using RPL4 as an endogenous control to normalize variations in mRNA expression. The PCR reactions were performed using Maxima SYBR Green/ROX qPCR Master Mix (Fermentas) into a CFX96 Real-Time System (Bio-Rad, Herefordshire, England). Reactions for qPCR contained 50 ng of each complementary DNA, plus 0.5 µM of each primer, 12.5 µL of 2x SYBR green/ROX qPCR Master Mix, and DNase/RNase free water to complete a final volume of 25 µL. The results were analyzed with the software CFX Manager 3.0 (Bio-Rad). The PCR conditions used for amplification and quantification were an initial denaturing stage (95 °C for 1 min), followed by 40 cycles of amplification (denaturing at 95 °C for 15 s; annealing at 56 °C for 15 s; and extension at 72 °C for 30 s); and a melting curve program (60 °C to 90 °C). Fluorescence was measured at the end of each cycle and every 0.2 °C during the melting program. Three-duplicate negative controls were used: qPCR reactions without DNA template, qPCR reactions with DNA template but no Sybr mix, qPCR reactions with DNA template but no primers. The melting curve of each specific qPCR product was analyzed to make sure that no primer dimers or nonspecific DNA products were quantitated. Results of quantitation of mRNA expression were analyzed according to comparative Ct method, expressed as $2^{-\Delta\Delta Ct}$ as described previously (Livak and Schittgen, 2001) and were normalized by RPL4 mRNA expression in each sample.

Statistical analysis

Analyses of variance of the data were performed on all variables according to the experimental design utilized. Three non-orthogonal contrasts were constructed to compare the effect of AT (TN100 vs. HS100), the supplemental effect of 20% DL-Met for HS pigs (HS100 vs. HS120), and the average effect of supplemental DL-Met for HS pigs with that of TN pigs (TN100 vs. HS120-HS140-HS160). Also, polynomial contrasts were constructed to analyze the linear or quadratic response of the HS pigs to supplemental DL-Met. Pearson correlation analyses were performed between the SC of free AA and the total intake of AA during the third week of the study, at the end of which blood samples were collected. Probability levels of $P \leq 0.05$, and $0.05 < P \leq 0.10$ were defined as significant differences and tendencies, respectively.

Table 3. Primers used for the quantitative PCR analyses of messenger RNA derived from amino acid transporters and ribosomal protein L4

mRNA	Primer	Sequence (5′→3′)	Amplicon size (bp)
B^0, Sus scrofa system B0 neutral amino acid transporter SLC6A19 mRNA, partial cds (DQ231579.1)			
	Forward	5′TCTGTCCACAACAACTGCGA3′	163
	Reverse	5′ACCCGTTGATAAGCGTCAG3′	
$b^{0,+}$, Sus scrofa solute carrier family 7 member 9 (SLC7A9), mRNA (NM_001110171.1)			
	Forward	5′CAAAACTGTCTGTGGGAGCC3′	385
	Reverse	5′GAGAGCACCTTGAGCATGTG3′	
y^+L, Sus scrofa solute carrier family 7 member 7 (SLC7A7), mRNA (NM_001110421.1)			
	Forward	5′GTGGGGAACATGATTGGCTC3′	244
	Reverse	5′CGATGATGAGGAGGGAGGTC3′	
RPL4, Sus scrofa ribosomal protein L4 mRNA, partial cds (DQ845176.1)			
	Forward	5′TGAGCTCTATGGCACTTGGC3′	239
	Reverse	5′GAATGGTGTTTCGGCGCATT3′	

Results

The average daily AT inside the HS room varied considerably within the same day ($P < 0.05$), from 29.0 °C to 36.3 °C (Figure 1). The highest AT records inside the HS room (>35 °C) was between 1300 and 1845 hours. In the TN room, the AT ranged from 20 °C to 25 °C, which is within the TN zone for the pigs used in the present study. The BT followed a pattern similar to that of AT (Figure 2) in both the TN and HS, but it was 1.0 °C to 1.9 °C higher in HS than in TN pigs ($P \leq 0.05$) from around 1200 to 2100 hours. The lowest BT was recorded during the early morning hours and the highest BT during the afternoon–evening (1430 to 2200) hours. All pigs remained healthy without apparent signs of digestive disorders such as diarrhea during the complete trial.

The effect of AT and DL-Met supplementation levels on the respiratory frequency differed between the times it was measured (Figure 3). At 0700 hours, the number of breaths per min of HS pigs fed the DL-Met supplemented diets increased ($P < 0.01$) and that of the HS100 pigs tended to increase ($P < 0.10$) in comparison with the TN100 pigs. At 1700 hours, the RF was higher in all HS pigs than in the TN100 pigs ($P < 0.01$). There was no effect of diet DL-Met level on the RF of HS pigs at any time. Overall, the RF of the HS pigs was higher at 1700 hours than at 0700 hours.

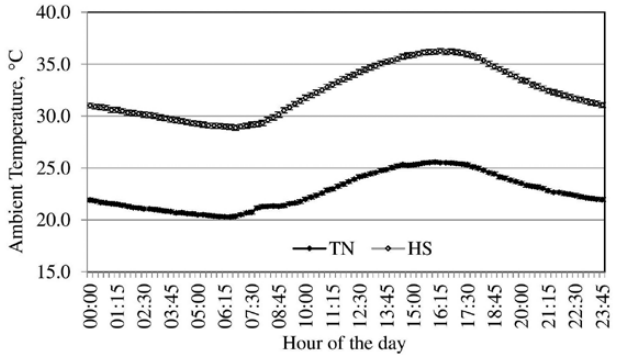

Figure 1. Ambient temperature recorded daily at 15-min intervals inside both the heat stress and the thermal neutral room with the aid of a hygrothermograph.

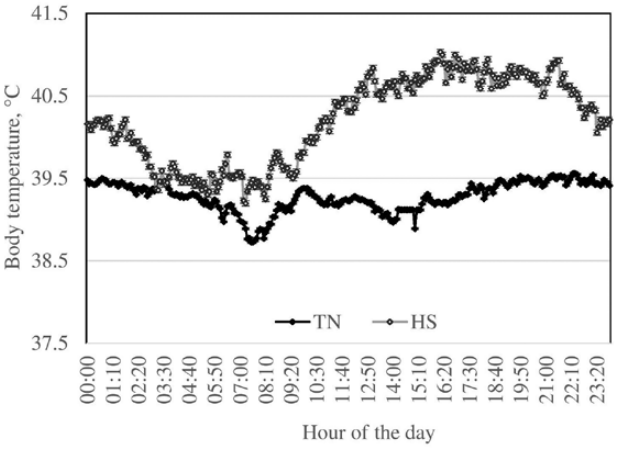

Figure 2. Body temperature of pigs recorded daily at 5-min intervals inside either the heat stress or the thermal neutral room with the aid of a thermograph implanted into the terminal ileum.

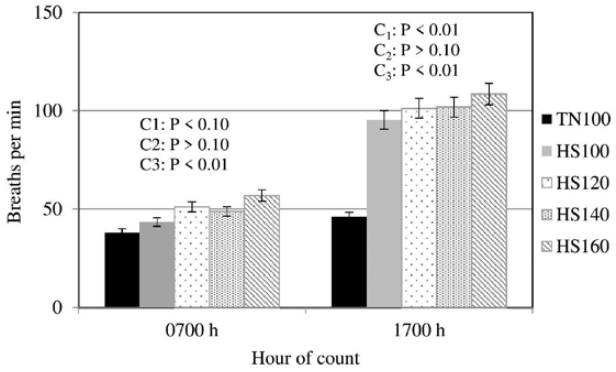

Figure 3. Respiratory frequency (breaths per min) of pigs housed inside either the heat stress or the thermal neutral room, recorded at 0700 and 1700 hours on days 16 and 18 of the trial. Each value is the average number of abdominal expansions per pig, counted twice at each time, 2 d, 8 pigs per treatment ($N = 32$). Contrasts: C_1, TN100 vs. HS100; C_2, HS100 vs. HS120; C_3, TN100 vs. HS120-140-160.

The performance of pigs was affected by both AT and DL-Met supplementation (Table 4). The ADG of the HS100 was lower than that of the TN100 and the HS120 pigs ($P < 0.01$); but it did not differ between the TN100 and the HS120 pigs ($P > 0.10$). The ADFI of the HS pigs with the DL-Met supplemented diets was lower than that of TN100 pigs ($P < 0.05$), although it did not differ among the TN100 and the HS100 pigs ($P > 0.10$). The G:F ratio was higher in the HS120 than in the HS100 pigs ($P < 0.05$). The calculated daily AA intake during week 3 of the trial when blood samples were collected (Table 5), with the exception of Met, was lower in HS-B than in TN-B pigs ($P < 0.05$). Also, as expected, Met and Lys intake linearly increased in HS pigs as result of the increased levels of both AA in the diet ($P < 0.05$).

The histomorphology of duodenum, jejunum, and ileum of pigs is presented in Table 6. In duodenum, the VH of TN100 pigs was greater than that of HS100 pigs and that of the HS pigs fed the diet supplemented with DL-Met ($P < 0.01$), but it did not differ ($P > 0.10$) among the HS100 and HS120 pigs. The VH in duodenum was greater for HS120-140-160 pigs compared to HS100 pigs. The CD in TN100 pigs was greater ($P < 0.01$) than that of HS pigs fed the diet either without (HS100) or with supplemental DL-Met (HS120, HS140, and HS160), but no difference was observed between HS pigs fed the diet either with or without supplemental DL-Met. The VH:CD ratio was larger in both the TN100 and HS100 pigs compared with HS pigs fed the DL-Met supplemented diets ($P < 0.05$). In jejunum, VH and VH:CD ratio were greater in TN100 pigs than in HS100 or the average in HS pigs fed the diets supplemented with DL-Met ($P < 0.05$). Crypt depth was influenced neither by AT nor supplemental DL-Met. In ileum, VH and VH:CD ratio were greater in TN100 pigs than in HS100 or the average in HS pigs fed the diets supplemented with DL-Met ($P < 0.05$). Crypt depth was lower in TN100 than the average in HS pigs fed the diets supplemented with DL-Met ($P < 0.05$).

The activities of antioxidant enzymes in serum are shown in Figure 4. Serum SOD, CAT, and GSH decreased in HS100 pigs ($P < 0.05$) in comparison with TN100 pigs, but those enzyme activities were higher in the HS120 pigs ($P < 0.01$)

Table 4. Performance of pigs exposed to either thermal neutral or heat stress conditions and fed diets with different supplemental DL-Met levels

Item [1]	Treatment[2]					Contrasts, *P*-value[3]			
	TN100	HS100	HS120	HS140	HS160	SEM	C_1	C_2	C_3
BW (d 0), kg	22.9	22.9	23.0	23.1	23.0	0.7	0.997	0.887	0.891
BW (d 21), kg	38.2	35.8	38.1	36.3	38.1	1.1	0.100	0.162	0.564
ADG, kg[a,b]	0.728	0.612	0.720	0.716	0.719	0.03	0.005	0.002	0.756
ADFI, kg	1.401	1.318	1.263	1.240	1.291	0.06	0.267	0.398	0.031
G:F[a]	0.522	0.474	0.568	0.563	0.562	0.03	0.202	0.025	0.195

[1]IBW, initial body weight; FBW, final body weight; ADG, average daily weight gain; ADFI, average daily feed intake; G:F, gain:feed ratio.
[2]TN100, thermal neutral pigs, 100% DL-Met requirement in diet; HS100, HS120, HS140, HS160, heat stress pigs, 100%, 120%, 140%, or 160% DL-Met requirement in diet.
[3]Contrast: C_1, TN100 vs. HS100; C_2, HS100 vs. HS120; C_3, TN100 vs. HS120-140-160.
[a]DL-Met levels in HS pigs: Linear, $P < 0.05$.
[b]DL-Met levels in HS pigs: Quadratic, $P < 0.10$.

Table 5. Intake (g/d) of essential amino acids by pigs exposed to thermal neutral or heat stress conditions and fed diets with supplemental levels of DL-Met during the 3rd week of the trial

Item	Treatment[1]					*P*-value[2]			
	TN100	HS100	HS120	HS140	HS160	SEM	C_1	C_2	C_3
Arg	16.6	14.1	14.2	14.1	15.1	0.8	0.036	0.943	0.017
His	6.8	5.8	5.8	5.7	6.0	0.3	0.038	0.986	0.011
Ile	11.2	9.6	9.5	9.5	10.0	0.5	0.036	0.915	0.011
Leu	19.8	16.8	16.7	16.6	17.5	1.0	0.036	0.942	0.017
Lys[a]	20.6	17.5	17.9	18.3	20.3	1.0	0.042	0.781	0.080
Met[a]	5.1	4.3	4.7	6.1	7.4	0.3	0.089	0.321	0.104
Phe	13.5	11.5	11.5	11.3	11.9	0.7	0.036	0.993	0.012
Thr	13.3	11.3	10.9	11.3	12.3	0.7	0.036	0.673	0.016
Trp	4.3	3.6	3.6	3.5	3.8	0.2	0.034	0.821	0.009
Val	13.9	11.8	13.7	13.7	12.5	0.8	0.036	0.911	0.012

[1]TN100, thermal neutral pigs, 100% DL-Met requirement in diet; HS100, HS120, HS140, HS160, heat stress pigs, 100%, 120%, 140%, or 160% DL-Met requirement in diet.
[2]Contrast: C_1, TN100 vs. HS100; C_2, HS100 vs. HS120; C_3, TN100 vs. HS120-140-160.
[a]Linear effect of dietary DL-Met levels for only HS pigs: $P < 0.01$.

when compared to the HS100 pigs ($P < 0.05$). The activity of GPX was higher in HS100 pigs than in the TN100 pigs ($P < 0.05$). On average, the activities of SOD and GSH were lower in all HS pigs fed the DL-Met-supplemented diet compared to TN100 pigs ($P < 0.05$). There was a linear increase in CAT activity and a linear decrease in GPX in response to increasing levels of DL-Met in diets for HS pigs ($P < 0.01$).

The relative expression of mRNA coding for the AA transporters $b^{0,+}$ and B^0 in jejunum (Table 7) was not affected by AT ($P > 0.10$) at the same DL-Met level (TN100 vs. HS100), but there was a linear increase of $b^{0,+}$ in the HS pigs because of the increased levels of supplemental DL-Met in the diet ($P < 0.05$). In ileum, the relative mRNA abundance for B^0 reduced in the HS pigs fed the diet either without (HS100) or with supplemental DL-Met, compared to the TN100 pigs ($P < 0.05$), but the abundance of mRNA coding for the cationic AA transporters $b^{0,+}$ and y+L was not affected neither by AT nor supplemental DL-Met level in the diet ($P > 0.10$).

The SC of free essential AA was differently affected by AT and the supplementation of DL-Met (Table 8). Serum concentrations of Ile, Leu, Lys, Phe, and Val were higher in HS100 than in TN100 pigs ($P < 0.01$). Compared to HS100, the SC of His, Leu, Lys, Phe, Thr, and Val decreased in HS120 pigs, but that of Met increased ($P < 0.05$); serum Arg, Ile, and Trp were not affected. On average, the SC of Lys was lower but that of Met was higher in HS pigs fed the DL-Met supplemented diets (HS120, HS140, and HS160) compared to the TN100 pigs ($P < 0.05$). The polynomial analyses of the SC of free essential AA considering only the HS pigs showed that Ile, Leu, and Val linearly decreased ($P < 0.01$), that of His, Lys, and Thr tended to decrease ($P < 0.10$), but Met linearly increased in response to the increased dietary levels of DL-Met ($P < 0.01$). Also, there was a quadratic response of serum Leu, Lys, Met, and Phe to the supplemental levels of DL-Met. The Pearson correlation coefficient between Met intake and SC of each free AA (Table 9) was highly positive ($r = 0.92$) and significant ($P < 0.05$) only for Met. But, the SC of Met was not correlated with the SC of the other essential AA.

The SC of free nonessential AA (Table 10) was not affected by AT (TN100 vs. HS100), except for Gln and Gly that were higher in pigs fed the HS100 diet ($P < 0.05$). Serum Ser tended to decrease in the HS120 pigs compared to the HS100 pigs ($P \leq 0.10$) but the other nonessential AA were not affected. The average SC of free Gln in the HS pigs fed

Table 6. Histomorphology of duodenum, jejunum and ileum of pigs (day 21) exposed to either thermal neutral or heat stress conditions and fed diets with different levels of supplemental DL-Met

Item	Treatment[1]					*P*-value[2]			
	TN100	HS100	HS120	HS140	HS160	SEM	C_1	C_2	C_3
Duodenum									
VH[a,b]	402	350	349	336	372	8.5	0.001	0.804	0.001
CD[a,c]	150	132	151	147	148	4.4	0.004	0.001	0.733
VH:CD[b]	2.77	2.70	2.43	2.43	2.60	0.08	0.518	0.020	0.002
Jejunum									
VH[a,b]	423	331	294	340	340	8.8	0.001	0.514	0.001
CD[a,b]	157	127	124	129	110	3.4	0.624	0.208	0.511
VH:CD [a]	2.77	2.68	2.47	2.77	3.18	0.09	0.001	0.124	0.001
Ileum									
VH[a,b]	320	252	308	322	305	7.7	0.001	0.340	0.001
CD[b]	118	102	141	112	134	0.07	0.028	0.926	0.009
VH:CD	2.80	2.55	2.39	2.95	2.32	4.5	0.019	0.001	0.032

[1]TN100, thermal neutral pigs fed 100% DL-Met requirement in diet; HS100, HS120, HS140, HS160, heat stress pigs fed 100%, 120%, 140%, or 160% DL-Met requirement in diet.
[2]Contrast: C_1, TN100 vs. HS100; C_2, HS100 vs. HS120; C_3, TN100 vs. HS120-140-160.
[a]DL-Met levels in HS pigs: linear, $P < 0.05$.
[b]DL-Met levels in HS pigs: quadratic, $P < 0.05$.
[c]DL-Met levels in HS pigs: quadratic, $P < 0.10$.

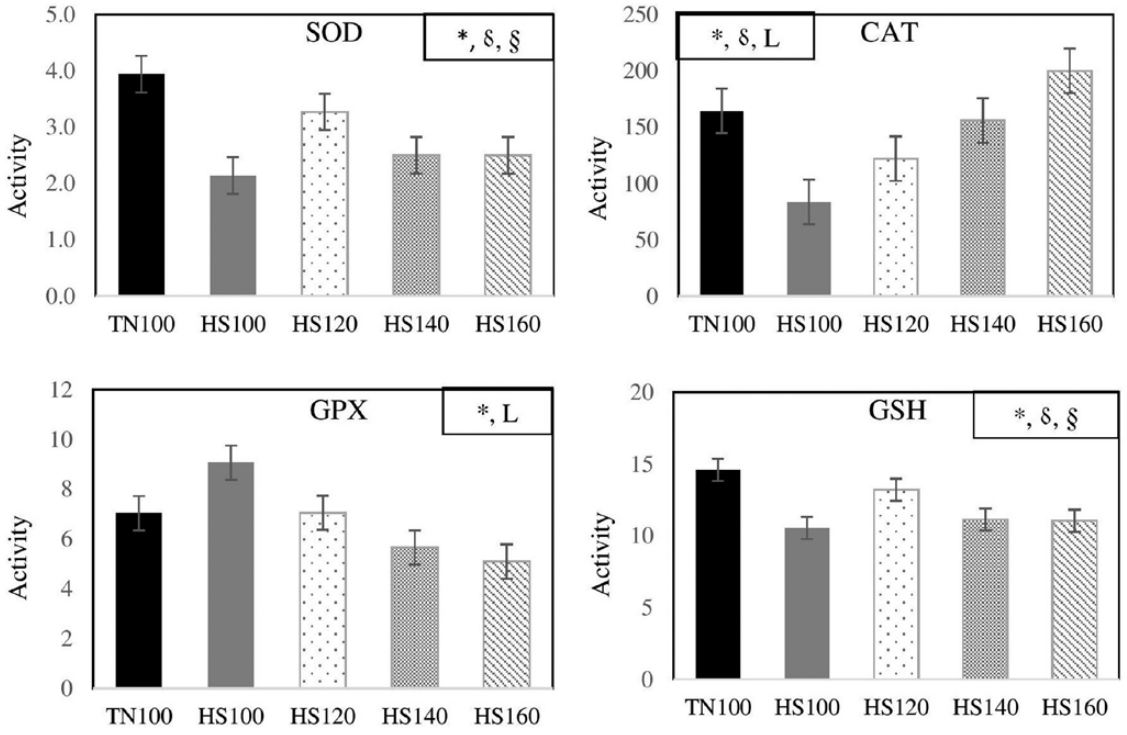

Figure 4. Activities of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and concentration of reduced glutathione in serum of pigs exposed to thermal neutral (TN) or heat stress (HS) conditions fed diets with 100, 120, 140, or 160 DL-Met levels. Mean ± SEM, $N = 6$. Statistical notations: *TN100 vs. HS100 ($P < 0.05$); δHS100 vs. HS120 ($P < 0.05$); §TN100 vs. HS120-HS140-HS160 ($P < 0.05$); L, lineal effect of supplemental DL-Met on HS pigs ($P < 0.05$).

the diet supplemented with DL-Met (HS120, HS140, and HS160) was higher than that in TN100 pigs ($P < 0.01$). There was a linear tendency to decrease serum Gly, Pro, and Ser in the HS pigs as the level of supplemental DL-Met in the diet increased ($P < 0.10$). Among the AA metabolites (Table 11), carnosine decreased, but homocysteine increased ($P < 0.05$), and 1-methyl.HIs and OH-Pro tended to increase ($P < 0.10$) in pigs fed the HS100 diet compared to those with the TN100 diet. On average, compared to pigs fed the TN100 diet, serum homocysteine decreased but phospho-Ser and α-NH_3-butyric

Table 7. Relative expression of the cationic amino acid transporters b0,+AT and y+L, and the neutral amino transporter B0AT2 in jejunum and ileum of pigs exposed to either thermal neutral or heat stress conditions and fed diets with supplemental levels of DL-Met (arbitrary units)

Item	Treatment[1]					*P*-value[2]			
	TN100	HS100	HS120	HS140	HS160	SEM	C_1	C_2	C_3
Jejunum									
B^0AT2	1	0.73	0.52	1.08	1.18	0.43	0.918	0.599	0.688
$b^{0,+}AT^a$	1	1.2	1.64	3.43	3.80	0.56	0.756	0.883	0.544
Ileum									
B^0AT2	1	0.3	0.44	0.36	0.34	0.34	0.009	0.413	0.013
$b^{0,+}AT$	1	1.44	0.82	0.99	0.85	0.39	0.359	0.123	0.707
y^+L	1	0.77	0.72	0.73	0.54	0.43	0.556	0.660	0.245

[1]TN100, thermal neutral pigs fed 100% DL-Met requirement in diet; HS100, HS120, HS140, HS160, heat stress pigs fed 100%, 120%, 140%, or 160% DL-Met requirement in diet.
[2]Contrast: C_1, TN100 vs. HS100; C_2, HS100 vs. HS120; C_3, TN100 vs. HS120-140-160.
[a]DL-Met levels in HS pigs: linear, $P < 0.05$.

Table 8. Serum concentrations (μg/mL) of free essential amino acids in pigs exposed to either thermal neutral or heat stress conditions and fed diets with supplemental levels of DL-Met

Item	Treatment[1]					*P*-value[2]			
	TN100	HS100	HS120	HS140	HS160	SEM	C_1	C_2	C_3
Arg	11.8	11.3	10.9	10.9	10.8	0.5	0.584	0.614	0.169
His	5.2	5.0	4.8	4.8	4.8	0.2	0.547	0.615	0.169
Ile	7.8	7.3	7.3	7.5	7.2	0.4	0.545	0.618	0.169
Leu	15.0	14.4	13.9	13.9	13.8	0.7	0.547	0.615	0.169
Lys[a]	13.9	13.3	13.6	14.4	15.1	0.7	0.578	0.751	0.529
Met[a]	3.9	3.8	4.4	5.2	5.8	0.2	0.641	0.050	0.542
Phe	14.9	14.3	13.8	13.8	13.7	0.7	0.547	0.615	0.169
Thr	8.5	8.2	7.9	7.9	7.8	0.4	0.547	0.618	0.169
Trp	2.5	2.4	2.3	2.3	2.3	0.2	0.547	0.615	0.169
Val	9.1	8.7	8.4	8.4	8.3	0.4	0.545	0.614	0.168

[1]TN100, thermal neutral pigs fed 100% DL-Met requirement in diet; HS100, HS120, HS140, HS160, heat stress pigs fed 100%, 120%, 140%, or 160% DL-Met requirement in diet.
[2]Contrast: C_1, TN100 vs. HS100; C_2, HS100 vs. HS120; C_3, TN100 vs. HS120-140-160.
[a]DL-Met levels in HS pigs: Linear, $P < 0.05$.

acid increased in HS pigs fed the DL-Met supplemented diets ($P < 0.05$).

Discussion

The effect of graded level of supplemental DL-Met in the diet on performance, antioxidant activity of several enzymes, the concentration of free AA in serum as well as the intestinal histo-morphology and relative expression of genes coding for AA transporters in pigs exposed to HS was evaluated in the present study. Signs of HS such as reduced feed intake and increased BT (Yu et al., 2010; Pearce et al., 2014) are common in pigs exposed to AT exceeding 33 °C. In multiple studies conducted at our lab, pigs exposed to AT above 35 °C consistently show similar or more severe HS signs (Morales et al., 2015, 2016a, 2016b, 2018; Cervantes et al., 2017). The HS pigs used in the present experiment were exposed to AT above 35 °C (up to 36.3 °C) for about 5 h every day that, compared to TN pigs, was correlated with the increased BT (up to 1.9 °C; 41.0 °C vs. 39.1 °C) and respiratory frequency (up to 2×; 102 vs. 46 breaths per min), respectively, indicating that these pigs experienced HS.

In the present study, the reduction in voluntary feed intake observed in HS pigs compared to the TN100 pigs was smaller than previously observed in this lab (Morales et al., 2014, 2018) and by other authors (Collin et al., 2001; Pearce et al., 2014), probably because the daily exposure of the HS pigs to high AT was for shorter periods of time compared to those reports. Nevertheless, the ADG of the HS100 pigs decreased 19% compared to TN100 pigs. Likewise, although the HS120 pigs consumed less feed, they had similar ADG to that of the TN100 pigs, and 20% higher ADG and feed efficiency than the HS100 pigs. This response was associated with a 17% increment in the consumption of DL-Met by the HS120 pigs, in comparison with the HS100 pigs. The consumption of the other essential AA did not differ among the HS120 and HS100 pigs. Hence, the improved growth rate and feed efficiency of the HS120 pigs (HS pigs fed a diet supplemented with 20% extra DL-Met) may indicate that Met was limiting in the diet given to the HS100 pigs, and that no more than 20% extra DL-Met is needed in the diets for HS pigs. Moreover, the 19% reduced ADG of the HS100 pigs compared to the TN100 pigs, despite the lack of difference in feed intake among them, suggests that factors related to the

Table 9. Pearson correlation coefficients (*r*) between total Met intake (MetI) or serum Met (MetS) and the serum concentration of free essential amino acids (*N* = 6; NS, not significant)

Serum amino acid	Amino acid intake			
	MetI		MetS	
	r	*P*-value	*r*	*P*-value
ArgS	0.17	NS	0.35	NS
HisS	0.03	NS	−0.16	NS
IleS	−0.26	NS	0.01	NS
LeuS	−0.14	NS	0.02	NS
LysS	0.03	NS	0.05	NS
MetS	0.92	0.023	—	—
PheS	−0.05	NS	0.21	NS
ThrS	−0.16	NS	−0.04	NS
TrpS	0.111	NS	0.14	NS
ValS	−0.22	NS	−0.06	NS

Table 10. Serum concentrations (μg/mL) of free non-essential amino acids in pigs exposed to either thermal neutral or heat stress conditions and fed diets with supplemental levels of DL-Met

Item	Treatment[1]					*P*-value[2]			
	TN100	HS100	HS120	HS140	HS160	SEM	C_1	C_2	C_3
Ala	72.40	90.18	93.19	82.88	81.83	7.69	0.115	0.784	0.139
Asp	3.44	4.10	4.90	3.68	3.72	0.46	0.321	0.221	0.223
Asn	9.49	10.02	9.72	8.74	8.83	0.74	0.620	0.777	0.650
Glu	19.19	24.93	34.01	20.29	23.76	3.87	0.305	0.100	0.139
Gln	44.68	68.29	72.36	58.02	59.31	5.55	0.006	0.608	0.008
Gly[a]	56.12	76.90	66.52	62.76	63.76	5.56	0.014	0.199	0.212
Pro[a]	67.91	80.92	82.75	73.87	67.62	6.07	0.142	0.833	0.340
Ser[a]	32.95	37.83	29.05	32.58	27.32	3.34	0.311	0.075	0.401
Tyr	25.03	24.40	25.49	23.47	23.19	2.31	0.850	0.741	0.718

[1]TN100, thermal neutral pigs fed 100% DL-Met requirement in diet; HS100, HS120, HS140, HS160, heat stress pigs fed 100%, 120%, 140%, or 160% DL-Met requirement in diet.
[2]Contrast: C_1, TN100 vs. HS100; C_2, HS100 vs. HS120; C_3, TN100 vs. HS120-140-160.
[a]Linear effect of dietary DL-Met levels in HS pigs: $P < 0.10$.

exposure of pigs to HS other than feed intake are involved in that response. Presumably, the diversity of functions that Met exerts in the cell, could have also contributed to both the reduced performance of HS100 pigs and the recovered performance of the HS120 pigs.

The increased BT of pigs exposed to HS (Pearce et al., 2014; Morales et al., 2018) seems to lower the supply of oxygen and nutrients to the small intestine (Wilson and Crandall, 2011; Ogoh et al., 2013) that in turn alters cell survivability and the integrity of the small intestine epithelia (Pearce et al., 2013) affecting the absorption of nutrients. The exposure of cells to HS also elevates the production of ROS (Kikusato and Toyomizu, 2013; Pardo and Seiquer, 2021) that may shift the balance between oxidants and antioxidants in favor of oxidants causing oxidative stress (Kurutas, 2016). The antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase are biomarkers of oxidative stress because they are important components of the defense system against ROS (Pardo and Seiquer, 2021). However, if oxidative stress exceeds the antioxidant capacity of the cell, it may lead to additional damage of the intestinal epithelia (Akbarian et al., 2016). Indeed, several authors reported damage to the intestinal epithelia of HS pigs linked to BT increments of up to 1.5 °C as evidenced by their shortened intestinal villi height (Liu et al., 2008; Pearce et al., 2013; Morales et al., 2021). In agreement with those reports, the villi height in duodenum, jejunum, and ileum of the HS100 pigs used in the present study decreased by 15% to 28% partially attributed to the increment in BT (up to 1.9 °C), compared to the TN100 pigs. This small intestine response was also associated with the substantial decrease in the activity of superoxide dismutase and catalase, as well as glutathione in the HS100 pigs. Hence, in agreement with previous reports, these data suggest that both increased BT and reduced activity of antioxidant enzymes in the HS100 pigs, are in part responsible for the alteration in their intestinal epithelia.

We hypothesized that Met might help to recover the intestinal villi height of HS pigs by improving the activity of the antioxidant system. Methionine functions as an antioxidant either in free form or as protein-bound Met. Moskovitz et al. (2001) reported that Met residues in cellular antioxidant proteins are highly susceptible to oxidation by ROS and that

Table 11. Serum concentrations (μg/mL) of amino acids metabolites in pigs exposed to either thermal neutral or heat stress conditions and fed diets with supplemental levels of DL-Met

Amino acid	Treatment[1]					*P*-value[2]		
	TN100	HS100	HS120	HS140	HS160	SEM	C1	C2
β-Ala	0.70	0.70	0.82	0.38	0.68	0.15	0.975	0.664
Carnosine	5.68	3.83	5.16	4.03	3.76	0.54	0.024	0.446
Citrulline	8.39	6.83	8.28	7.46	6.99	0.93	0.248	0.493
Ethanol-NH_2	2.73	2.26	1.94	1.86	3.11	0.40	0.409	0.918
Homocysteine	0.10	2.92	1.08	1.65	0.77	0.52	0.001	0.007
1-Methyl-His	1.42	3.87	2.67	2.69	2.51	0.95	0.079	0.265
3-Methyl-His	0.81	1.08	0.99	0.88	0.90	0.11	0.085	0.216
OH-Lys	0.90	0.80	1.04	0.68	0.94	0.20	0.749	0.732
OH-Pro	7.74	10.66	11.89	10.46	11.14	1.21	0.100	0.720
Ornithine	19.82	17.26	17.81	15.45	15.94	2.17	0.413	0.735
Phospho-Ser	1.44	1.46	1.77	1.63	1.65	0.09	0.849	0.049
Sarcosine	1.51	5.91	4.86	4.54	3.42	2.01	0.134	0.487
Taurine	14.65	12.94	14.46	14.19	15.27	1.44	0.407	0.316
Urea	261.7	252.1	261.8	265.9	213.6	20.3	0.740	0.834
α-NH_3-butyric acid	1.63	1.31	2.23	2.30	2.97	0.34	0.511	0.006

[1]TN100, thermal neutral pigs fed 100% DL-Met requirement in diet; HS100, HS120, HS140, HS160, heat stress pigs fed 100%, 120%, 140%, or 160% DL-Met requirement in diet.
[2]Contrasts: C1, TN100 vs. HS100, C2, HS100 vs. HS120-140-160.

the oxidized Met can be reduced by a sulfoxide reductase. Also, proteins in Complex I of the respiratory chain where most of ROS production takes place are abundant in Met (Schindeldecker and Moosmann, 2015). Moreover, Met is precursor for the synthesis of glutathione (Bauchart-Thevret et al., 2009), an important component of the cellular antioxidant system that functions in association with glutathione peroxidase (Kurutas, 2016). These reports help to explain the improved activity of superoxide dismutase and catalase as well as GSH in the HS120 pigs in comparison with the HS100 pigs of the present experiment and partially confirm our hypothesis. Met is also involved in other important pathways related to intestinal morphology. First, Met functions as a methyl-group donor through S-Adenosyl-Met for the synthesis of polyamines, which are important cell proliferation stimulators (Wu, 2009) and for the methylation of DNA that is critical in the rapidly proliferating small intestine epithelia (Miousse et al., 2017). Supplemental Arg, which also functions as precursor for the polyamines synthesis (Wu, 2009), improved intestinal villi height of HS pigs (Morales et al., 2021). However, supplementing 20% or more Met above the requirement of growing pigs did not help to recover the small intestine villi height observed in the TN100 pigs, suggesting that factors other than oxidative stress, S-Adenosyl-Met, and DNA methylation are also responsible. Pearce et al. (2013) reported shortened intestinal villi in both TN pair-fed and HS pigs in comparison with TN pigs fed ad libitum suggesting that reduced feed intake might be responsible for that response. Thus, there is still need to further test that hypothesis.

The reduced villi height in duodenum, jejunum, and ileum of the HS100 pigs, compared to the TN100 pigs, might be expected to affect the expression of genes coding for the synthesis of AA transporters (Wang et al., 2009). Actually, the reduced villi height in ileum was associated only with a reduced abundance of the mRNA coding for the neutral AA transporter B^0, which is involved in the absorption of Met. On the other hand, the lack of effect of dietary DL-Met supplementation levels on intestinal morphology was also associated with the lack of effect on the expression of mRNA coding for the synthesis of B^0. We examined the expression of the cationic $b^{0,+}$ and the neutral B^0 transporters because $b^{0,+}$ transports the first limiting AA (Lys), and B^0 transports Met and other neutral AA exported from the enterocyte in exchange for Lys during absorption (Bröer, 2008). In agreement with a previous report (Cervantes et al., 2016), the exposure of pigs to HS conditions (AT < 36 °C) similar to those in the present experiment did not affect the expression of the cationic AA transporters $b^{0,+}$ and y^{+L}, in opposition to (Morales et al., 2014) who reported a reduction in the expression of transporter $b^{0,+}$ in jejunum of pigs exposed to severe HS (up to 42 °C). These data suggest the effect of HS on the mRNA expression of AA transporters depends on the severity of the HS exposure.

The SC of free AA reflects mostly the small intestine AA absorption and the uptake of AA by the cells (Reverter et al., 2000). The AA absorption depends on several factors including the content and form (free or protein-bound) of dietary AA (Yen et al., 2004; Cervantes et al., 2017) as well as the function of the small intestine epithelia (Wang et al., 2009). In the present experiment, coinciding with these reports, free Met and Lys were the only AA whose SC increased in a linear fashion in response to the increased supplementation level of DL-Met in the diet. Interestingly, the SC of free Lys, Ile, Leu, Phe, and Val increased 16% to 26% in the HS100 pigs compared with the TN100 pigs (Figure 5), although the abundance of mRNA coding for the cationic AA (Lys) transporters $b^{0,+}$ and y^{+L} (Bröer, 2008) did not differ among these pigs, or even more, the abundance of mRNA for the neutral AA (Ile, Leu, Phe, Val) transporter B^0 (Bröer, 2008) decreased in the

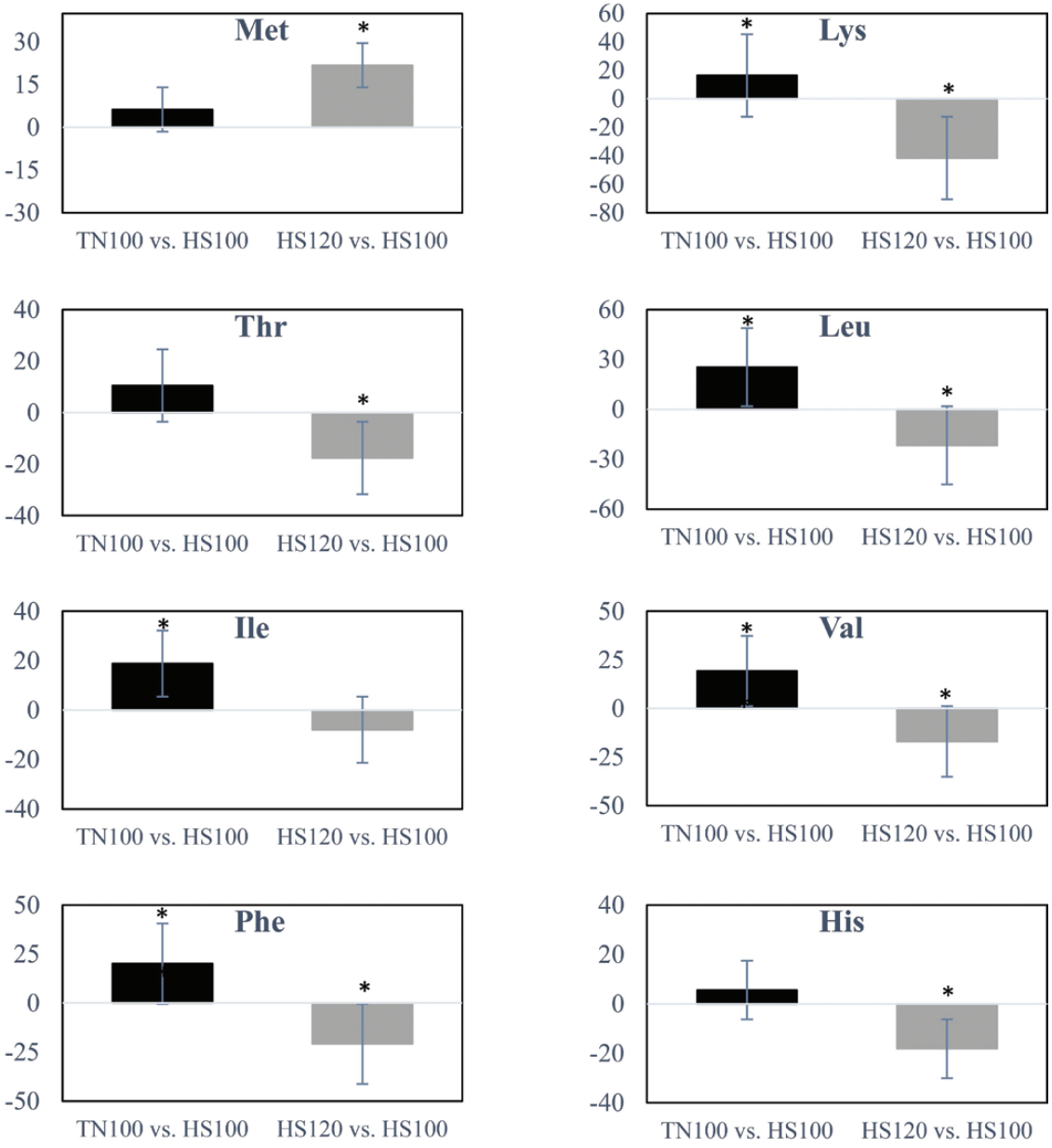

Figure 5. Relative changes (%) in the serum concentrations of free amino acids in TN100 (TN100 vs. HS100) and HS120 (HS100 vs. HS120) pigs in relation to the serum concentrations in HS100 pigs. Mean ± SEM, $N = 6$. *$P < 0.05$.

HS100 pigs. The majority of each absorbed AA function as building blocks (Wu, 2009) for protein synthesis, thus it is reasonable to believe that the synthesis of the skeletal muscle proteins affects the uptake of serum AA by the cells (Davis and Fiorotto, 2009). Methionine, likewise, is indispensable for the synthesis of all tissue proteins (Brosnan and Brosnan, 2007) and is third limiting AA in cereal–soybean meal diets for pigs (NRC, 2012). Hence, the increased SC of free Lys, Ile, Leu, Phe, and Val combined with the decreased daily weight gain of the HS100 pigs, compared with the TN100 pigs, suggest that Met may become first limiting in diets for growth of HS pigs. These serum AA increments may indicate a reduction in their cellular uptake, presumably limited by Met because serum Met remained without change among the HS100 and TN100 pigs and particularly because Lys was considered first limiting in the control diet for TN pigs. However, compared to the HS100 pigs, the decreased SC of free Lys, Thr, Leu, His, Phe, and Val in the HS120 pigs (Figure 5) suggest that their muscle cells might have increased the uptake of these AA to sustain the increased weight gain and feed efficiency, supporting the assumption that Met, at the NRC (2012) recommendation, became first limiting in the control diet for HS pigs. The lack of effect of further DL-Met supplementation levels on weight gain and feed efficiency regardless of the increased SC of Met suggest that adding 20% of DL-Met above the requirement may help HS pigs to sustain a growth performance comparable to that of TN pigs.

Changes in the SC of carnosine and homocysteine seem to be related to the exposure of pigs to HS and the DL-Met supplemental level in the diet. Carnosine has antioxidant activity due to its capability to attenuate cellular oxidative stress and to inhibit the intracellular formation of reactive-oxygen species (Reddy et al., 2005), thus the decreased SC of carnosine in the HS100 pigs was associated with the decreased activity

of antioxidant enzymes. Homocysteine is an intermediate in the formation of the methyl donor S-Adenosyl-L-Met (SAM) and results after deadenosylation and donating the methyl group to DNA, RNA, AA, proteins, among other molecules (Kumar et al., 2017). According to these authors, homocysteine remethylation to reform Met via enzymatic pathways is dependent on folate and vitamin B_{12}. The SC of homocysteine in the HS100 pigs increased about 30-fold as compared to the TN100 pigs but it decreased around 60% in HS pigs fed the diets supplemented with extra DL-Met. Probably HS100 pigs either increased the methylation of nucleic acids and proteins associated with the increased expression of genes involved in the cellular response to HS and oxidative stress, or these pigs likely consumed insufficient amounts of those B vitamins. Although α-amino-*n*-butyric is formed from Thr and Met, there is no clear explanation for its 25% SC decrease in the HS100 pigs compared to the TN100 pigs, followed by its increase (up to 3-fold) in HS pigs fed diets supplemented with DL-Met.

In conclusion, the exposure of pigs to HS has a strong effect on body temperature and performance, which appears to be associated with alterations in the morphology of the small intestine epithelia, the antioxidant status, respiration rate, and the concentration of free AA in serum. The supplementation of 20% DL-Met above its requirement to diets for growing TN pigs seems to help to restore the performance and some physiological variables of HS pigs, as compared to TN pigs. However, further DL-Met increments above 20% in the diet are of no further benefit for the HS pigs.

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Conflict of Interest Statement

The authors declare no real or perceived conflicts of interest.

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