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ORIGINAL ARTICLE

Performance, serum amino acid concentrations and expression of selected genes in pair-fed growing pigs exposed to high ambient temperatures

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Summary

Heat stress (HS) depresses pig performance mainly because of appetite reduction, although other factors involved in the cellular availability of nutrients may also contribute to that depression. An experiment was conducted with twelve pair-fed pigs (30.3 ± 2.7 kg BW) to examine the effect of severe HS (up to 45 °C) on the expression of genes coding for two cationic amino acid (AA) transporters ($b^{0,+}$AT and CAT-1), leptin, heat-shock protein (Hsp-90) and myosin in several tissues; serum concentrations (SC) of AA; and performance. There were two treatments: Comfort, pigs housed at an average temperature of 22 (±2) °C; and HS, pigs housed in a similar room with no climate control, where temperature was raised up to 45 °C. All pigs received the same wheat–soybean meal diet and had similar daily feed intake. Comfort pigs had a higher daily gain and better gain/feed ratio than HS pigs ($p < 0.05$). The expression of $b^{0,+}$AT in jejunum and liver, that of myosin in the *Semitendinosus* muscle, and leptin in adipose tissue was lower, but CAT-1 in jejunum and liver, and Hsp-90 in liver was higher in HS pigs. The SC of Lys and Met in HS pigs were around 55% and 20%, respectively, of that in Comfort pigs ($p < 0.05$). In conclusion, HS affects the expression of cationic AA transporters, myosin, Hsp-90, leptin; the SC of Lys and Met; and the performance of pair-fed pigs. These results suggest that HS-related changes in gene expression affect the performance of pigs beyond the effect caused by the reduction in voluntary feed intake.

Keywords pigs, heat stress, gene expression, serum amino acids, performance

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Introduction

Heat stress (HS) is defined as a series of alterations in the physiology, metabolism and behaviour of animals exposed to high ambient temperature (AT; Horowitz et al., 2004). The heat increment resulting from digestion and metabolism of nutrients may additionally increase the stress caused by high AT (Le Bellego et al., 2002). In pigs, the effect of HS on performance has been thoroughly studied, and the reports (e.g. Huynh et al., 2005) show that voluntary feed intake (VFI) is greatly depressed in pigs exposed to HS, which in turn reduces their weight gain (ADG). The reduced VFI directly provokes a decrease in the availability of nutrients for growth. Leptin is known to participate in the regulation of VFI in pigs housed under thermoneutral conditions (Barb et al., 2001), but it is not clear whether leptin also participates in the reduction in VFI in pigs exposed to high AT.

Heat-stressed pigs activate compensatory mechanisms to prevent or at least minimize cell damage and protect vital organs (Kregel and Moseley, 1996). These include, besides the reduction in VFI, changes in vital functions such as respiratory frequency and heart rate (Huynh et al., 2005) and increases in the synthesis of heat-shock proteins (Hsp), which help newly formed proteins to attain their proper folding and function (Kiang and Tsokos, 1998). These changes may increase the need for energy and amino acids (AA), which could be deviated from growth in HS pigs. Also, according to Sonna et al. (2002), HS induces alterations in cell membrane function and cellular proliferation that may affect the transport of nutrients across the cell membrane. Indeed, Yu et al. (2010) reported that HS causes marked damage to the tips of the intestinal villi, shorter villus height and crypt depth in jejunum, the main absorption site for AA and other nutrients in pigs. Hence, pigs exposed to HS may have

a reduced number of absorptive cells and fewer nutrient transporters per intestinal villi. The cationic AA transporters are the most critical in pigs because Lys is the first limiting AA in most feed ingredients. Cationic AA are transported at high affinity by the Na-independent systems $b^{0,+}$AT and CAT-1 (Majumder et al., 2009). The $b^{0,+}$AT system is mainly expressed in epithelial cells and exchanges Leu for Lys, so intestinal absorption of cationic AA by $b^{0,+}$AT is coupled with the Leu efflux (Torras-Llort et al., 2001); CAT-1 is ubiquitously expressed and most abundant in non-epithelial cells (Hatzoglou et al., 2004). However, to our knowledge, there is no report regarding the expression of genes coding for cationic AA transporters in pigs exposed to high AT.

It is speculated that these modifications can reduce the availability of nutrients for growth, beyond the reduction caused by the lowered VFI. Therefore, we hypothesize that severe HS affects the availability of nutrients for animal growth by depressing the digestive and absorptive functions and the deviation of nutrients from growth to sustain the compensatory mechanisms induced by HS. Thus, the objectives of this study were as follows: (i) to evaluate the effect of very high AT causing severe HS (up to 45 °C) on the expression of genes coding for the cationic AA transporter $b^{0,+}$AT and CAT-1 as well as leptin, Hsp-90 and myosin in several tissues and on the serum concentrations (SC) of AA in pair-fed growing pigs; and (ii) to determine whether the performance depression of pigs exposed to high AT is also provoked by factors other than the lowered VFI.

Materials and methods

Location, animals and diets

An experiment was conducted in July 2010, when AT fluctuates from 25 (average low) to 42 °C (average high). There were two treatments: Comfort, pigs housed in a temperature-controlled room with the thermostat set up at 22 (±2) °C; and HS, pigs housed in a similar room but with no climate control. The maximum and minimum AT inside the HS room were measured on a daily basis during the conduction of this study (July 2010) using a digital device (AcuRite Digital Thermometer; Lake Geneva, WI, USA); on average, these AT were 42.0 and 26.5 °C respectively. Twelve cross-bred (Landrace × Hampshire × Duroc) pigs [30.3 ± 2.7 kg initial body weight (BW)] were used to form six pairs of pigs; each pair was of same sex and litter, and similar initial BW. One pig of each pair was assigned at random to the Comfort room and the other to the HS room. There were six replicates (three barrows and three gilts) per treatment. Pigs were individually housed in raised floor metabolism pens (1.2 m wide, 1.2 m long and 1.0 m high) equipped with a stainless steel self-feeder, a nipple water drinker and iron mesh floor. Average daily weight gain (ADG), feed intake and the gain/feed ratio were calculated on a weekly basis. The average BW of the pigs at the end of the 19 days study was 41.1 ± 2.90 kg. The pigs used in the experiment were cared for in accordance with the guidelines established in the Official Mexican Regulations on Animal Care (NOM-062-ZOO-1999, 2001).

All pigs were pair-fed a wheat (845 g/kg)–soybean meal (120 g/kg) diet supplemented with 0.26% L-Lys and 0.05% L-Thr, which was formulated to meet or exceed the requirements of the indispensable AA, vitamins and minerals for the 20–50 kg pigs (NRC, 1998). The diets were analysed for CP (method 984.13; AOAC, 2006) and AA content (method 982.30; AOAC, 2006). The CP and essential AA composition of the diet (g/kg) was as follows: CP, 163.0; Arg, 8.7, His, 4.0; Ile, 6.3; Leu, 11.6; Lys, 8.9; Met, 2.3; Phe, 7.6; Thr, 5.4; Val, 7.6. The diet contained 13.8 MJ of ME per kg. Pigs within each pair of pigs had a similar feed intake, although it was different among pairs of pigs. The feed intake of all pigs was restricted to approximately 95% of the *ad libitum* VFI of HS pigs and adjusted every week during the study. This amount of feed was approximately 36% lower than the estimated VFI of Comfort pigs at this BW. Meals were offered two times a day, at 7:00 and 19:00 hours. All pigs were adapted to the metabolism pens and trained to consume their daily meals within 30 min or less, during 5 days previous to the start of the performance trial, and had unlimited access to water.

Tissue collection

At the end of the experiment, all pigs were euthanized by electrical stunning and exsanguination for tissue collection, 2.5 h after their last feeding at 7:00 hours. The corpses were immediately eviscerated, and mucosal samples scratched from middle jejunum (approximately 0.5 g) were collected into 2-ml micro tubes. Samples from liver, subcutaneous dorsal adipose tissue, *Longissimus dorsi* (LDM) and *Semitendinosus* (STM) muscles (approximately 0.5–1.0 g) were also collected. All samples were immediately frozen in liquid nitrogen and stored at −82 °C until analysis. The total collection process took no longer than 8 min to maximize the quality of the extracted RNA. In addition, blood samples (approximately 10 ml) were

collected from the carotid artery to analyse the SC of free AA, right before the pigs were euthanized.

Total RNA extraction and purification

Samples of jejunum mucosa, liver, adipose tissue, LDM and STM were treated to extract total RNA by pulverization into liquid nitrogen and homogenized into Trizol reagent (Invitrogen, Carlsbad, CA, USA) as reported by Méndez et al. (2011). Purified RNA was eluted with 30 μl of RNase-free distilled water and stored at −82 °C. The concentration of total RNA was determined spectrophotometrically (Helios β; Thermo Electron, Rochester, NY, USA) at 260 nm, and purity of RNA was assessed 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 2.0:1.0 (Sambrook and Russell, 2001).

Reverse transcription and quantitative PCR

Approximately 2 μg of total RNA was treated with 1 U of DNase I (1 U/μl; Invitrogen) and 6 μl of 5× reverse transcription buffer in a 30 μl reaction completed with DEPC-treated water; the reaction was carried out for 15 min at room temperature and another 15 min at 70 °C to stop the reaction. Reverse transcription was initiated with DNase-treated RNA samples, adding 1 μl of random primers (150 ng/μl, Invitrogen) and 1 μl of dNTPs solution (10 μM each), reaction mixture was incubated 5 min at room temperature and then chilled on ice for 1 min; 3 μl of DTT (0.1 M), 1 μl of ribonuclease inhibitor (40 U/μl; RNase OUT, Invitrogen) and 2 μl of 5× reverse transcription buffer were added to the reaction and incubated at 42 °C during 2 min to stabilize the reaction before adding 1 μl of reverse transcriptase enzyme (200 U/μl; RT-Superscript III, Invitrogen). Reverse transcription reaction mixture was incubated at 42 °C during 50 min, and at 70 °C for 15 min, then chilled on ice to stop the reaction. cDNA samples were quantified spectrophotometrically and diluted into a final concentration of 50 ng/μl.

Specific primers for $b^{0,+}$AT (*SLC7A9*), CAT-1 (*SLC7A1*), leptin (*LEP*), Hsp-90 (*Hsp-90AA1*) and myosin heavy chain of isoform IIB (*MYH4*) mRNAs and the 18S rRNA were designed according to their published sequences at the GenBank (Table 1). A housekeeping 18S rRNA (GenBank AY265350) was used as an endogenous control to normalize variations in mRNA. Before starting, end-point PCR was carried out to standardize the amplification conditions for each pair of primers and to confirm the specificity of the PCR products related to its mRNA, a sample of every PCR product was sequenced at the Davis Sequencing Facility (Davis, CA, USA). Sequencing results revealed that the products for $b^{0,+}$AT, CAT-1, leptin and Hsp-90, and 18S rRNA have 100% homology with their corresponding expected sequences acquired from the virtual template sequences reported in the GenBank.

Expression of genes (relative mRNA abundance) coding for $b^{0,+}$AT, CAT-1, leptin, Hsp-90 and myosin was estimated by quantitative PCR (qPCR) assays, using the Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Glen Burnie, MD, USA) into a Chromo 4-DNA Engine with the MJ OPTICON MONITOR 3.1 software (Bio-Rad, Herefordshire, UK). The equipment was calibrated with a standard curve using the 18S rRNA cloned into a TOPO vector 4.0, from which a calibrator's cDNA was produced. Relative

Table 1 Primers used for the real-time PCR analyses of cDNA derived from the amino acid transporters $b^{0,+}$AT and CAT-1, myosin, Hsp-90 and leptin mRNA, and the 18S ribosomal RNA

Gene	Primer	Location (bp) on the template	Sequence	Amplicon size (bp)
$b^{0,+}$AT (*SLC7A9*) (GenBank: EF127857)	Forward	1–19	5′CGGAGAGAGGATGAGAAGT3′	562
	Reverse	545–562	5′GCCCGCTGATGATGATGA3′	
Cationic amino acid transporter-1, CAT-1 (*SLC7A1*) (GenBank: AY371320)	Forward	4239–4258	5′GTCGGTTGCAAAGACCATTT3′	329
	Reverse	4548–4567	5′GAGCGGTGCTGACAACAGTA3′	
Myosin, heavy chain 4 (*MYH4*) (GenBank: NM_001123141)	Forward	4582–4599	5′AGATTTCTGACCTGACTG3′	340
	Reverse	4904–4921	5′TCTCCCTCCATCTTCTTC3′	
Heat-shock protein (*Hsp-90AA1*), Sus scrofa 90-kDa (GenBank: NM_213973.1)	Forward	991–1010	5′GATCACTTGGCTGTGAAGCA3′	470
	Reverse	1441–1460	5′TTGAGGGAAACCATCTCGTC3′	
Leptin, (*LEP*) Sus scrofa leptin mRNA (GenBank: NM_213840.1)	Forward	879–899	5′ACGTTGAAGCCGTGCCCATCT3′	307
	Reverse	1168–1185	5′GGCAAGGGGCAGCTCTTGGA3′	
18S rRNA (GenBank: AY265350)	Forward	236–255	5′GGCCTCACTAAACCATCCAA3′	295
	Reverse	511–530	5′TAGAGGGACAAGTGGCGTTC3′	

standard curve methods for each specific mRNA were obtained using known concentrations of five 100-fold serial dilutions of the cDNA, and the linear range for target mRNA quantification was established. Reaction mixture for qPCR contained 50 ng of cDNA, 0.5 μM of each specific primer, 12.5 μl of 2× SYBR (green/ROX qPCR Master Mix) and DNase/RNase-free water to complete a final volume of 25 μl. The PCR conditions used for the amplification and quantification were an initial denaturing stage (95°C for 1 min), followed by 45 cycles of amplification (denaturing at 95°C for 30 s, annealing at 56°C for 15 s and extension at 72°C for 30 s) and a melting curve program (60–90°C). Fluorescence was measured at the end of every cycle and every 0.2 °C during the melting program. The relative mRNA abundance was normalized to the relative 18S abundance by calculating the mRNA:18S relative abundance ratios (Liao et al., 2009).

Analysis of free AA in serum

Blood samples were centrifuged at 500 *g*, 4 °C for 10 min to separate serum from blood cells, and serum was deproteinized with a Millipore Ultrafree-MC 10 000 NMWL Filter Unit (Millipore, Bedford, MA, USA) at 5000 *g*, 4 °C for 30 min (Sunde et al., 2003). The supernatant filtrate was derivatized with Waters AccQÆTag reagent. The analysis of AA was performed by HPLC (method 982.30E; AOAC, 2006) with post-column ninhydrin derivatization and with the use of a fluorescence detector.

Statistical analysis

Statistical analyses were performed by independent sample *t*-tests using SAS (Statistical Analysis System 9.1; SAS Institute, Cary, NC, USA). Multiple correlation analyses between the expression of genes coding for $b^{0,+}$AT, CAT-1, myosin and the SC of some AA were also performed. Probability levels of $p \leq 0.05$ and $0.05 < p \leq 0.10$ were defined as significant differences and tendencies respectively.

Results

All pigs consumed their daily rations within 15 min and did not show any clinical sign of disease during the whole experiment. The performance results are presented in Table 2. Pigs exposed to severe HS had lower ADG ($p = 0.014$) and gain/feed ratio ($p = 0.013$) as compared to Comfort pigs. Daily feed intake, as planned, was similar among treatments.

Table 2 Performance of pigs exposed to either severe heat stress (up to 45 °C) or thermo-neutral conditions (22 °C) fed a wheat–soybean meal diet

	Treatment			
Item	Comfort	Heat stress	SEM	p-value
Average weight gain, kg/day	0.634	0.569	0.015	0.014
Feed intake, kg/day	1.10	1.10	–	–
Gain/feed ratio	0.576	0.517	0.014	0.013

The results of the relative gene expression are presented in Table 3. The expression of the $b^{0,+}$AT gene was markedly lower in jejunum ($p = 0.011$) and liver ($p = 0.002$) of pigs exposed to high AT, and it was barely expressed in both muscles. In contrast, the expression of CAT-1 in jejunum ($p = 0.033$) and liver ($p = 0.003$) was higher in HS pigs. The expression of genes coding for CAT-1 in LDM and STM was not affected. Pigs exposed to high AT expressed less myosin mRNA in STM ($p = 0.006$), but it was not affected in LDM ($p = 0.195$). The abundance of Hsp-90 mRNA in liver was higher ($p = 0.004$), and leptin mRNA in adipose tissue was lower ($p = 0.007$) in HS pigs.

The serum AA values are presented in Table 4. The SC of Lys ($p = 0.026$) and Met ($p = 0.007$) were lower in HS pigs. In contrast, the SC of Ile ($p = 0.025$), Leu ($p = 0.014$) and Val ($p = 0.005$) were higher and that of Phe tended to be higher ($p = 0.094$) in HS pigs. The SC of Arg, His and Thr were not affected by the housing temperature of pigs ($p > 0.10$).

The results of the multiple correlation analyses are presented in Table 5. The expression of $b^{0,+}$AT in

Table 3 Relative abundance of mRNA coding for $b^{0,+}$AT, CAT-1, myosin, Hsp-90 and Leptin in several tissues of pigs under severe heat stress (up to 45 °C) or thermo-neutral conditions (22 ± 2 °C) fed a wheat–soybean meal diet (arbitrary units; ratio of mRNA:18S rRNA)

		Treatment			
Gene	Tissue	Comfort	Heat stress	SEM	p-value
$b^{0,+}$AT (*SLC7A9*)	Jejunum	2.983	0.450	0.544	0.011
	Liver	2.240	0.542	0.002	0.002
CAT-1 (*SLC7A1*)	Jejunum	0.0489	0.1538	0.030	0.033
	Liver	0.0009	0.0047	0.0007	0.003
	LDM	0.0084	0.0061	0.0011	0.171
	STM	0.0050	0.0023	0.011	0.126
Myosin (*MYH4*)	LDM	4.140	2.731	0.718	0.195
	STM	1.059	0.1986	0.281	0.006
Hsp-90 (*Hsp-90AA1*)	Liver	0.025	0.300	0.049	0.004
Leptin (*LEP*)	Adipocyte	0.700	0.065	0.126	0.007

Table 4 Serum concentrations (μM) of nine essential amino acids in pigs exposed to either severe heat stress (up to 45 °C) or thermo-neutral conditions (22 ± 2 °C) fed a wheat–soybean meal diet

	Treatment			
Indispensable amino acid	Comfort	Heat stress	SEM	p-value
Arginine	567	552	62	0.864
Histidine	689	513	108	0.290
Isoleucine	341	470	31	0.025
Leucine	397	592	40	0.014
Lysine	580	306	66	0.026
Methionine	95	17	14	0.007
Phenylalanine	214	310	34	0.094
Threonine	639	483	120	0.394
Valine	667	1401	119	0.005

jejunum was negatively correlated with CAT-1 in jejunum and liver ($p < 0.01$) and the SC of Leu ($p = 0.05$), but positively correlated with myosin expression in STM ($p < 0.01$). Also, the expression of $b^{0,+}$AT in jejunum tended to be positively correlated with the expression of $b^{0,+}$AT in liver ($p = 0.10$), myosin in LDM ($p = 0.06$) and the SC of Lys ($p = 0.10$). The expression of CAT-1 in jejunum was positively correlated with that of CAT-1 in liver ($p < 0.01$) and tended to be positive with that of $b^{0,+}$AT in liver ($p = 0.09$); CAT-1 in liver was negatively correlated with myosin in LDM ($p = 0.05$). The SC of Leu was negatively correlated with the SC of Lys ($p < 0.01$) and positively correlated with CAT-1 expression in jejunum.

Table 5 Coefficients and p-values for correlation analyses between the relative expression of genes coding for $b^{0,+}$AT, CAT-1 and myosin (Myo) in the *Longissimus* (LDM) and *Semitendinosus* (STM) muscles; and serum concentration (SC) of Lys and Leu

	$b^{0,+}$AT Jejunum	CAT-1 Jejunum	CAT-1 Liver	Leu SC
$b^{0,+}$AT Jejunum	ns*	ns	ns	$r = -0.70$ ($p = 0.05$)
$b^{0,+}$AT Liver	$r = 0.62$ ($p = 0.10$)	$r = 0.64$ ($p = 0.09$)	ns	ns
CAT-1 Jejunum	$r = -0.85$ ($p < 0.01$)	ns	ns	$r = 0.71$ ($p = 0.05$)
CAT-1 Liver	$r = -0.82$ ($p < 0.01$)	$r = 0.85$ ($p < 0.01$)	ns	ns
Myosin LDM	$r = 0.69$ ($p = 0.06$)	ns	$r = -0.70$ ($p = 0.05$)	ns
Myosin STM	$r = 0.86$ ($p < 0.01$)	ns	ns	ns
Lys SC	$r = 0.60$ ($p = 0.10$)	ns	ns	$r = -0.89$ ($p < 0.01$)

*ns: Correlation coefficient not significant ($p > 0.10$).

Discussion

The aim of this study was to determine whether severe HS affects the expression of selected genes related to the availability of cationic AA, the synthesis of muscle protein and performance of growing pigs. Thus, the study was conducted in July 2010, under practical conditions in which HS pigs were exposed to natural diurnal variations in AT. During the conduction of this study, as shown in Fig. 1, there were 11 days with maximum AT ranging from 43 °C to 45 °C (usually from 14:00 to 19:00 hours each day) and 13 days ranging from 40 °C to 42 °C; average low AT ranged from 20 °C to 26 °C (usually from 3:00 to 7:30 hours each day). Relative humidity ranged from 25% to 56%, with an average of 44%. According to Collin et al. (2001), pigs exposed to an AT equal to or higher than 29 °C develop HS. In this study, HS pigs were under severe HS every day, alternating periods of very high with others of moderate AT. All HS pigs exhibited a marked increase in respiratory frequency and heart rate during 12 or more hours every day (data not shown).

This study was also aimed to determine that VFI reduction is not the only factor depressing performance of pigs under severe HS. The pair-fed approach was used to achieve this objective; feed intake of Comfort pigs was limited to the average VFI of HS pigs. All pigs consumed the same diet and similar amounts, which was equivalent to 64% of the estimated VFI of Comfort pigs (NRC, 2012). The ADG and the gain/feed ratio of HS pigs were 11.4% lower than those of Comfort pigs, even though all pigs had similar feed intake. In agreement, Collin et al. (2001) found that VFI decreased 47% but the ADG decreased 51% in growing pigs exposed to 35 °C, as compared to those exposed to 19 °C. Similar tendencies were reported with young pigs under mild heat stress (McGlone et al., 1987). Collectively, these results suggest that factors other than reduced feed consumption, including the molecular and physiological mechanisms activated by the animal to cope with HS, are also responsible for their performance depression. The VFI of pigs is regulated by different factors including leptin, which is an appetite suppressor produced by several cell types, especially by adipocytes (Margetic et al., 2002). Morera et al. (2012) reported an increase in leptin concentration and gene expression in C57BL/6 mice exposed to moderate chronic HS (35 °C) for 5 days. In our study, the expression of the leptin gene in HS pigs was approximately one-tenth as compared to that in Comfort pigs. This discrepancy

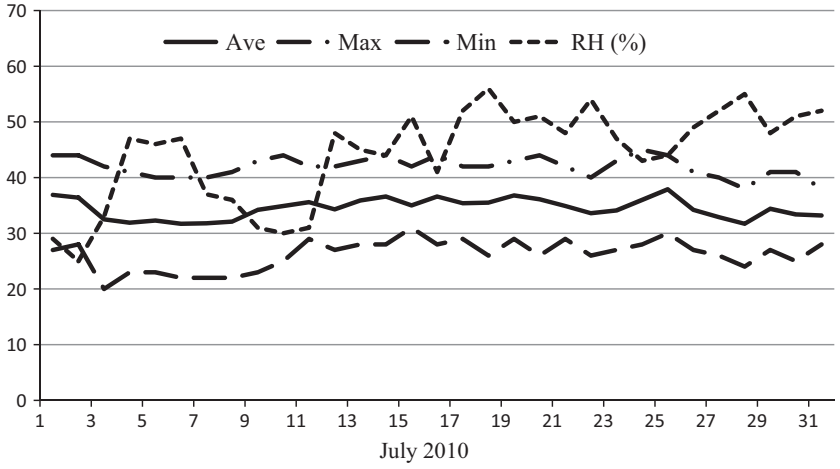

Fig. 1 Average (Ave), maximum (Max) and minimum (Min) temperatures (°C) and relative humidity (RH,%) registered inside the heat-stress room during July 2010.

may be attributed to differences in the type of animal used as experimental model; we used growing lean pigs, whereas Morera et al. (2012) used obese mice. Thus, our results might suggest that leptin may not be involved in the VFI reduction in lean pigs exposed to extremely high AT.

Heat stress may impair the intestinal absorptive activity of organisms by altering their intestinal cell function. Yu et al. (2010) reported a marked damage to the tips of the intestinal villi, shorter villus height and crypt depth in jejunum of mini pigs exposed to 40 °C for 5 h/day, for 10 consecutive days. In our study, pigs were repeatedly exposed to extremely high AT for 5 h/day or more, during 19 days, which may imply a reduced number of absorptive cells and lower abundance of nutrient transporters in HS pigs. The abundance of the $b^{0,+}$AT mRNA in jejunum and liver of HS pigs was approximately 15% and 24%, respectively, as compared to Comfort pigs. The low expression in jejunum might be explained as the result of the decreased proliferation of absorptive intestinal cells observed by Yu et al. (2010). The abundance of CAT-1 mRNA in jejunum and liver of HS pigs was approximately threefold and fivefold higher, respectively, as compared to Comfort pigs. These results are in agreement with Fernandez et al. (2003), who reported that rat cells stressed with high levels of cytosolic calcium increase CAT-1 expression by coordinated stabilization of the mRNA and increased mRNA translation. This may partially explain the difference of CAT-1 mRNA abundance in jejunum and liver between HS and Comfort pigs.

Based on the assumption that expression of genes coding for AA transporters represents fully their activity (Dave et al., 2004), the reduced $b^{0,+}$AT mRNA abundance in jejunum of HS pigs may suggest that the cellular availability of Lys, the first limiting AA in most feed ingredients for growth, is significantly lowered in HS pigs. Indeed, although there were no differences in feed intake in this study, the SC of Lys in HS pigs was around 50% of that in Comfort pigs. This response supports the assumption that the expression of AA transporters represents fully their activity. Moreover, this response supports the hypothesis that HS pigs had a reduced intracellular availability of Lys for growth; Lys was first limiting in the experimental diet of this study. Lys and Leu are the two most abundant AA in muscle proteins (Mahan and Shields, 1998), but Leu content in the experimental diet was above the requirement. Therefore, the reduced ADG and gain/feed ratio of HS pigs can be partially explained by the decreased availability of Lys. The higher SC of Leu in HS pigs is explained by the fact that $b^{0,+}$AT functions as antiporter (Torras-Llort et al., 2001) exchanging Leu for Lys (Broer, 2008). Thus, assuming the lower $b^{0,+}$AT mRNA abundance is reflected by a lower abundance of the transporter in jejunum, the reduced Lys SC in HS pigs may indicate a decreased efflux of Leu and could explain the higher SC of Leu in these pigs. The higher SC of Ile, Val and Phe in HS pigs may be similarly explained because these are also neutral AA. The expression of the transporter for Met, third limiting AA, was not analysed in this study, but the lower Met SC could suggest that HS also affects the expression of its transporter.

Type IIB fibres account for approximately 80% of the total fibres in some pig muscles (Davoli et al., 2003) and are extensively expressed in LDM and STM (Lefaucheur et al., 2004). In STM, abundance of mRNA myosin in Comfort pigs was around fivefold greater as compared to HS pigs. This response was related to the expression of $b^{0,+}$AT in jejunum and the serum Lys values: there was a highly positive correlation between the expression of genes coding for $b^{0,+}$AT in jejunum and myosin in STM ($r = 0.86$) or LDM ($r = 0.69$). Furthermore, the lower abundance of myosin mRNA observed in HS pigs coincides with their reduced ADG and poor gain/feed ratio. There seems to be an association between the growth rate of pigs, the expression of genes for $b^{0,+}$AT in jejunum and myosin and the low SC of Lys observed in this study. Thus, the low myosin mRNA abundance in HS pigs may also contribute to their performance depression.

Heat stress induces a rapid increase in the synthesis of the Hsp family (Lacetera et al., 2006). These are the most abundant proteins in the cell, and their function consists in assisting the non-covalent assembly of recently synthesized proteins to facilitate the correct folding (Gething and Sambrook, 1992). The abundance of Hsp-90 mRNA in HS pigs of the present study was 12-fold higher than in Comfort pigs. Similar results were published by Theodorakis and Morimoto (1987) in heat-stressed as compared to non-stressed cells. These results suggest that HS pigs may use an important amount of nutrients to sustain the synthesis of these proteins instead of using them for muscle growth and might partially contribute to the depressed performance of HS pigs, beyond the reduction in VFI.

In summary, severe heat stress provoked by high ambient temperatures affected the mRNA abundance of $b^{0,+}$AT and CAT-1 in jejunum and liver, myosin in the *Semitendinosus*, Hsp-90 in liver and leptin in adipose tissue, as well as the performance of pair-fed pigs. The average daily gain of heat-stressed pigs was 11.4% lower than that of Comfort pigs; also, the voluntary feed intake and average daily gain of stressed pigs were approximately 43% and 33% lower, respectively, than the NRC (2012) estimated values. Thus, factors other than reduced voluntary feed intake contributed with around 33% of the reduction in the average daily gain of heat-stressed pigs. Alterations in the expression of cationic amino acid transporters, myosin and Hsp-90, the availability of Lys, besides the molecular mechanisms activated by the cell as a response to HS, are suggested to be among those factors.

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References

AOAC, 2006: *Official Methods of Analysis*, 18th edn. Association of Official Analytical Chemists, Gaithersburg, MD, p. 2000.

Barb, C. R.; Hausman, G. J.; Houseknecht, K. L., 2001: Biology of leptin in the pig. *Domestic Animal Endocrinology* **21**, 297–317.

Broer, S., 2008: Amino acid transport across mammalian intestinal and renal epithelia. *Physiological Reviews* **88**, 249–286.

Collin, A.; van Milgen, J.; Le Dividich, J., 2001: Modelling the effect of high, constant temperature on food intake in young growing pigs. *Animal Science* **72**, 519–527.

Dave, M. H.; Schulz, N.; Zecevic, M.; Wagner, C. A.; Verreyet, F., 2004: Expression of heteromeric amino acid transporters along the murine intestine. *Journal of Physiology* **558**, 597–610.

Davoli, R.; Fontanesi, L.; Cagnazzo, M.; Scotti, M.; Buttazzoni, L.; Yerle, M.; Russo, V., 2003: Identification of SNPs, mapping and analysis of allele frequencies in two candidate genes for meat production traits: the porcine myosin heavy chain 2B (MYH4) and the skeletal muscle myosin regulatory light chain 2 (HUMMLC2B). *Animal Genetics* **34**, 221–225.

Fernandez, J.; Lopez, A. B.; Wang, C.; Mishra, R.; Zhou, L.; Yaman, I.; Snider, M. D.; Hatzoglou, M.; Hatzolgou, M., 2003: Transcriptional control of the arginine/lysine transporter, cat-1, by physiological stress. *Journal of Biological Chemistry* **278**, 50000–50009.

Gething, M. J.; Sambrook, J., 1992: Protein folding in the cell. *Nature* **355**, 33–45.

Hatzoglou, M.; Fernandez, J.; Yaman, I.; Closs, I., 2004: Regulation of cationic amino acid transport: the story of the CAT-1 transporter. *Annual Review of Nutrition* **24**, 377–399.

Horowitz, M.; Eli-Berchoer, L.; Wapinski, I.; Friedman, N.; Kodesh, E., 2004: Stress-related genomic responses during the course of heat acclimation and its association with ischemic-reperfusion cross-tolerance. *Journal of Applied Physiology* **97**, 1496–1507.

Huynh, T. T. T.; Aarnink, A. J. A.; Verstegen, M. W. A.; Gerrits, W. J. J.; Heetkamp, M. J. W.; Kemps, B.; Canh, T. T., 2005: Effects of increasing temperatures on physiological changes in pigs at different relative humidities. *Journal of Animal Science* **83**, 1385–1396.

Kiang, J. G.; Tsokos, G. C., 1998: Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. *Pharmacology & Therapeutics* **80**, 183–201.

Kregel, K. C.; Moseley, P. L., 1996: Differential effects of exercise and heat stress

on liver HSP70 accumulation with aging. *Journal of Applied Physiology* **80**, 262–277.

Le Bellego, L.; van Milgen, J.; Noblet, J., 2002: Effect of high temperature and low-protein diets on the performance of growing-finishing pigs. *Journal of Animal Science* **80**, 691–701.

Lefaucheur, L.; Milan, D.; Ecolan, P.; Le Callennec, C., 2004: Myosin heavy chain composition of different skeletal muscles in Large White and Meishan pigs. *Journal of Animal Science* **82**, 1931–1941.

Liao, S. F.; Vanzant, E. S.; Harmon, D. L.; McLeod, K. R.; Boling, J. A.; Matthews, J. C., 2009: Ruminal and abomasal starch hydrolysate infusions selectively decrease the expression of cationic amino acid transporter mRNA by small intestinal epithelia of forage-fed beef steers. *Journal of Dairy Science* **92**, 1124–1135.

Mahan, D. C.; Shields, R. G. Jr, 1998: Essential and nonessential amino acid composition of pigs from birth to 145 kilograms of body weight, and comparison to other studies. *Journal of Animal Science* **76**, 513–521.

Majumder, M.; Yaman, I.; Gaccioli, F.; Zeenko, V. V.; Wang, C.; Caprara, M. G.; Venema, R. C.; Komar, A. A.; Snider, M. D.; Hatzoglou, M., 2009: The hnRNA binding proteins hnRNP L and PTB are required for efficient translation of the Cat-1 arginine/lysine transporter mRNA during amino acid starvation. *Molecular and Cellular Biology* **29**, 2899–2912.

Margetic, S.; Gazzola, C.; Pegg, G. G.; Hill, R. A., 2002: Leptin: a review of its peripheral actions and interactions. *International, Journal of Obesity and Related Metabolic Disorders* **26**, 1407–1433.

McGlone, J. J.; Stansbury, W. F.; Tribble, L. F., 1987: Effects of heat and social stressors and within-pen weight variation on young pig performance and agonistic behavior. *Journal of Animal Science* **65**, 456–462.

Méndez, V.; Avelar, E.; Morales, A.; Cervantes, M.; Araiza, A.; González, D., 2011: A rapid protocol for purification of total RNA for tissues collected from pigs at a slaughterhouse. *Genetics and Molecular Research* **10**, 3251–3255.

Morera, P.; Basirico, L.; Hosoda, K.; Bernabucci, U., 2012: Chronic heat stress up-regulates leptin and adiponectin secretion and expression and improves leptin, adiponectin and insulin sensitivity in mice. *Journal of Molecular Endocrinology* **48**, 129–138.

Norma Oficial Mexicana (NOM-062-ZOO-1999). 2001: Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio. In: M.L.I. Ochoa (ed.). *Diario Oficial de la Federación*, Norma Official Mexicana, México (DF), México, pp. 1–58.

NRC, 1998: *Nutrient Requirements of Swine*, 10th revised edn. National Academy Press, Washington, DC.

NRC, 2012: *Nutrient Requirements of Swine*, 11th revised edn. National Academy Press, Washington, DC.

Sambrook, J.; Russell, D. W., 2001: *Molecular Cloning: A Laboratory Manual*, 3rd edn. Cold Spring Harbor Laboratory Press, New York.

Sonna, L. A.; Fujita, J.; Gaffin, S. L.; Lilly, C. M., 2002: Effects of heat and cold stress on mammalian gene expression. *Journal of Applied Physiology* **92**, 1725–1742.

Sunde, J.; Kiessling, A.; Higgs, D.; Opstvedt, J.; Venturini, G.; Rungruangsaktorrissen, K., 2003: Evaluation of feed protein quality by measuring plasma free amino acids in Atlantic salmon (*Salmo salar* L.) after dorsal aorta cannulation. *Aquaculture Nutrition* **9**, 351–360.

Theodorakis, N. G.; Morimoto, R. I., 1987: Posttranscriptional regulation of hsp7O expression in human cells: effects of heat shock, inhibition of protein synthesis, and adenovirus infection on translation and mRNA stability. *Molecular and Cellular Biology* **7**, 4357–4368.

Torras-Llort, M.; Torrents, D.; Soriano-García, J. F.; Gelpí, J. L.; Estévez, R.; Ferrer, R.; Palacín, M.; Moretó, M., 2001: Sequential amino acid exchange across $b^{0,+}$- like system in chicken brush border jejunum. *Journal of Membrane Biology* **180**, 213–222.

Yu, J.; Yin, P.; Liu, F.; Cheng, G.; Guo, K.; Lu, A.; Zhu, X.; Luan, W.; Xu, J., 2010: Effect of heat stress on the porcine small intestine: A morphological and gene expression study. *Comparative Biochemistry and Physiology* **156**, 119–128.