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Effect of Heat Stress on the Expression of HSP70, UCP3 and CYP450 Genes in Liver; *Longissimus Dorsi* and *Semitendinosus* Muscle of Growing Pigs

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Abstract: High Ambient Temperature (AT) provokes Heat Stress (HS) in animals, which is characterized by increased body temperature, Reactive Oxygen Species (ROS) production and cell damage. Uncoupling Proteins (UCP) may contribute to dissipate body heat, cytochrome P450 (CYP3A4) reduces ROS and Heat Shock Proteins (HSP) prevent protein denaturalization. This study analyzed the expression of HSP70, UCP3 and CYP3A in liver, *Longissimus Dorsi* (LD) and *Semitendinosus* (ST) muscles of pigs exposed to HS conditions. A 21-d experiment was conducted with 18 pigs (32.6±3.2 kg body weight) divided into 3 treatments: (1) HS, pigs exposed to natural AT (29.5-37.2°C) fed *ad libitum*; (2) TNad, thermoneutral conditions (24.0±2.0°C) fed *ad libitum*; (3) TNpf, thermoneutral fed same amount as HS pigs. Hepatic expression of HSP70 in HS pigs was 4-fold higher than in TNad pigs ($p=0.039$); no differences were observed between HS and TNpf, or between TNad and TNpf pigs ($p>0.10$). There were no differences in HSP70 expression in both muscles ($p>0.10$) because of HS or feed intake. Expression of UCP3 in LD and ST did not differ ($p>0.10$) between treatments; neither the expression of CYP3A in liver was affected by HS, or feed intake level ($p>0.10$). Apparently, pigs became adapted to HS because expression of mitochondrial UCP3 and CYP3A was not affected after 21 d of HS exposure and the increased HSP70 expression in liver could have helped cells to maintain their proteins integrity.

Keywords: Heat Stress, Heat Shock Protein, Uncoupling Protein, CYP3A, Pig

Introduction

Heat Stress (HS) occurs when Ambient Temperature (AT) and relative humidity exceed the Thermo Neutral (TN) zone of organisms leading to a series of physiological and behavioral responses. Heat stress pigs increase their respiratory frequency and heart rate to dissipate more body heat (Wilson and Crandall, 2011) and consume less feed to decrease body heat production (Bernabucci *et al*., 2010). Nonetheless, pigs exposed to severe HS increase Body Temperature (BT) up to 2.0°C (Pearce *et al*., 2013; Morales *et al*., 2016), which might result in cell death (Rhoads *et al*., 2013).

Body heat receptors appear to sense temperature changes at cellular level to maintain BT balance and prevent cell death. Heat Shock Proteins (HSP) are inducible proteins that prevent protein denaturalization under high AT (Katschinski, 2004). HSP70 is highly conserved in cells (Bakau and Horwich, 1998), whose expression is associated with a thermo tolerance cellular response (Kregel, 2002) and the presence of Reactive Oxygen Species (ROS) (Kregel, 2002). HS increases ROS production in mitochondria (Mujahid *et al*., 2007). Uncoupling Proteins (UCP) are mitochondrial anion-carriers that generate heat by dissipating the mitochondrial proton gradient, uncoupling respiration from ATP synthesis (Palmieri, 1994). UCP2 and UCP3 also attenuate the mitochondrial ROS production and protect against ROS-induced cellular damage (Mookerjee *et al*., 2010). The cytochrome P450s (CYPs) are proteins responsible for drugs metabolism (Duester, 1996), although it has been observed also that mouse

hepatocytes increase expression of the P450 isoform CYP3A4 under HS conditions (Li *et al*., 2012). Although these studies were conducted under highly controlled artificial lab conditions, we hypothesize that pigs exposed to natural HS conditions and different feed intake level will differently express genes coding for those proteins.

The objective of this study was to analyze the expression of HSP70, UCP3 and CYP3A in liver, *Longissimus Dorsi* (LD) and *Semitendinosus* (ST) muscle of pigs exposed to TN or natural HS conditions and different feed intake level.

Materials and Methods

Animals, Housing and Diets

All pigs in this experiment were cared for in accordance with the guidelines established in the Official Mexican Regulations on Animal Care (NOM-062-ZOO-1999, 2001). The study was conducted in Northwestern Mexico during summer time when AT fluctuates every day from 25 to 42°C. Eighteen crossbred (Landrace x Hampshire x Duroc) pigs with an initial body weight of 32.6±3.2 kg were randomly assigned to one of three treatments based on sex (3 males and 3 females), litter and body weight. Treatments were: HS, pigs exposed to high natural ambient temperature (housed in a room with no temperature control) and fed *ad libitum*; TNad, pigs exposed to thermo-neutral conditions (housed inside an air-conditioned room with the thermostat set at 24±2°C) and fed *ad libitum*; TNpf, thermo-neutral pigs pair-fed with the HS pigs. There were six replicates per treatment. All 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. Ambient temperature inside each room was recorded every 15 min during the study with the aid of a Higrothermograph (Thermotracker HIGRO; iButtonLink LLC, Whitewater, WI, USA).

All pigs were fed the same diet based on wheat (905 g/kg) and soybean meal (65 g/kg) supplemented with 0.48% L-Lys and 0.13% L-Thr, vitamins and minerals, which met the NRC (2012) requirements for pigs within the body weight range of 25 to 50 kg. The diet contained 14.0% CP and 10.1 MJ of NE per kg. The feed intake of HS pigs was restricted to 95% of their *ad libitum* feed intake recorded the previous week, which was approximately 20% lower than the voluntary feed intake of TNad pigs. This amount of feed was adjusted every week during the study. The TNpf pigs were pair-fed with HS pigs; these pigs were fed two times a day at 0700 and 1900 h in two equal meals. All pigs were adapted to the metabolism pens and trained to consume their daily meals within 30 min or less, during 7 days before the trial started. Purified water was available to all pigs during the study. The experiment lasted 21 d; the average body weight of pigs at the end of the study was 44.2, 49.2 and 43.6 kg for HS, TNad and TNpf, respectively.

Collection of Tissue Samples

At the end of the experiment, all pigs were sacrificed by electrical stunning and exsanguination. Immediately after slaughter, samples (0.5 g) of liver, *Longissimus Dorsi* (LD) and *Semitendinosus* (ST) muscles were collected into 2 ml micro tubes. Also, samples of blood and intestinal epithelia were collected to analyse the expression of amino acid transporters in the small intestine and the concentration of free AA in serum; these data are already published (Morales *et al*., 2016). All samples were immediately frozen in liquid nitrogen and stored at -80°C until lab analysis.

Total RNA Extraction and Purification

All tissue samples were treated to extract total RNA by the Trizol reagent (Invitrogen, Corp., Carlsbad, CA, USA), as reported previously (Méndez *et al*., 2011). Purified RNA was then eluted with RNase-free water and stored at -80°C. 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). 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). Approximately 2 µg of total RNA were treated with 1 U of DNase I (1 U μL^{-1}; Invitrogen) and reverse transcription was performed using random hexamers as previously described (García *et al*., 2015). The complementary DNA samples were quantified and diluted into a final concentration of 50 ng/µl.

Quantitative PCR (qPCR)

Specific primers for HSP70, UCP3 and CYP3A mRNA and 18S rRNA gene were designed according to their published sequences at the GenBank (Table 1). End point PCR were carried out to standardize the amplification conditions for each pair of primers and in order to confirm the specificity of the PCR products related to its mRNA, a sample of each PCR product was purified and sequenced at the GENEWIZ (South Plainfield, NJ, USA). Sequencing results revealed that the products for HSP70, UCP3, CYP3A and 18S rRNA showed 100% homology with their corresponding expected sequences acquired from the virtual template sequences reported in GenBank.

Table 1: Primers used for the quantitative PCR analyses of messenger RNA derived from uncoupled protein (UCP3), heat shock protein 70 (HSP70), cytochrome P450 (P450) and 18S ribosomal RNA from pig

mRNA	Primer	Location (bp) on the template	Sequence	Amplicon (bp)
Sus scrofa heat shock protein 70 (HSP70), mRNA (GenBank: NM_001123127.1)				
	Forward	289-307	GCCCTGAATCCGCAGAATA	152
	Reverse	440-423	TCCCCACGGTAGGAAACG	
Sus scrofa uncoupling protein 3 (UCP3) mRNA, nuclear gene encoding mitochondrial protein, complete cds. (GenBank: AF128837.1)				
	Forward	820-839	GAACTGTGCCGAGATGGTGA	281
	Reverse	1100-1081	AGCTTCCCAAGCGCAAAAAG	
Sus scrofa cytochrome P450 3A (CYP3A) mRNA, complete cds. (GenBank: AF424780.1)				
	Forward	1185-1204	GGTGCCAGTCTTCGTGCTTC	288
	Reverse	1472-1451	AGAACGACAGGTTTTTCCGTTG	
Sus scrofa 18s ribosomal RNA complete sequence (GenBank: AY265350)				
	Forward	236-255	GGCCTCACTAAACCATCCAA	295
	Reverse	530-511	TAGAGGGACAAGTGGCGTTC	

The expression of mRNA codding for HSP70, UCP3 and CYP3A was analyzed by quantitative PCR (qPCR) assays and 18S rRNA gene was used as an endogenous control to normalize variations in mRNA expression. Expression of HSP70, UCP3 and CYP3A was analyzed in the LD and ST muscles and in liver. PCR reactions were performed by duplicate using the Maxima SYBR Green-ROX qPCR Master Mix 2X (Fermentas, Inc. USA) into a CFX96 Real-Time System (Bio-Rad, Herefordshire, England) and results were analyzed with the software CFX Manager 3.0 (Bio-Rad). Reactions for qPCR contained 50 ng of cDNA, 0.5 μM of each specific primer, 12.5 μL of 2x SYBR green/ROX qPCR Master Mix and nuclease-free water to complete a final volume of 25 μL. PCR conditions used for the amplification and quantification were an initial denaturing stage of 95°C for 5 min, followed by 40 cycles of amplification (denaturing at 95°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 45 s) and a melting curve program (60 to 90°C). Fluorescence was measured at the end of every cycle and every 0.2°C during the melting program. Three-replicate negative controls were used: qPCR reactions without DNA template; qPCR reactions with DNA template but no SYBR Mix; and 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 non-specific DNA products were quantitated. Results of quantitation of mRNA expression were analyzed according to comparative Ct method, expressed as $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001) and normalized by 18S ribosomal RNA expression in each sample.

Statistical Analysis

Expression of mRNA for HSP70, UCP3 and CYP3A was analyzed using SAS (Statistical Analysis System 9.1; SAS Institute, Cary, NC, USA), according to a completely randomized design. Three contrasts were constructed to test the effects of exposing pigs to different ambient temperature at similar feed intake (C_1, HS *vs*. TNpf), different feed intake under TN conditions (C_2, TNad *vs*. TNpf) and different ambient temperature and feed intake (C_3, HS *vs*. TNad). Probability levels of $p<0.05$ and $0.05\leq p<0.10$ were defined as significant and tendencies respectively.

Results

Feed Intake and Ambient Temperature

Feed intake, as designed in the present experiment, did not differ between the HS and TNpf pigs (1.2 kg/d). However, as expected, the TNad pigs consumed more feed (1.47 kg/d) than the other two groups of pigs ($p<0.05$). Ambient temperature inside the room with no air-conditioning fluctuated every day from 29.5°C (around 0630 h) to 37.2°C (around 1630 h); the average temperature was 33.1±2.6°C (Fig. 1). In contrast, AT inside the air-conditioned room fluctuated from 24.0 to 28.1°C, with an average of 25.9±1.4°C.

mRNA Expression

The relative expression of mRNA coding for HSP70 in response to the exposure of pigs to different AT and feed intake level, varied depending on the analyzed tissue (Fig. 2). In liver, the expression of HSP70 was higher in pigs exposed to HS than in pigs exposed to TN conditions and fed *ad libitum* ($p=0.039$). However, no difference in HSP70 expression was observed between HS pigs and TN pigs having similar feed intake ($p=0.543$), or between TN pigs fed either *ad libitum* or restricted ($p=0.110$). Regarding the relative expression of HSP70 in muscle, no effect of ambient temperature at similar feed intake (HS *vs*. TNpf) was observed in LD ($p=0.528$) and ST ($p=0.676$), or feed intake level in pigs exposed to TN conditions (TNad *vs*. TNpf) in LD ($p=0.588$) and ST ($p=0.936$). The HSP70 expression in the LD ($p=0.927$) and ST ($p=0.732$) muscles of HS pigs did not differ from that of TN pigs fed *ad libitum* (HS *vs*. TNad).

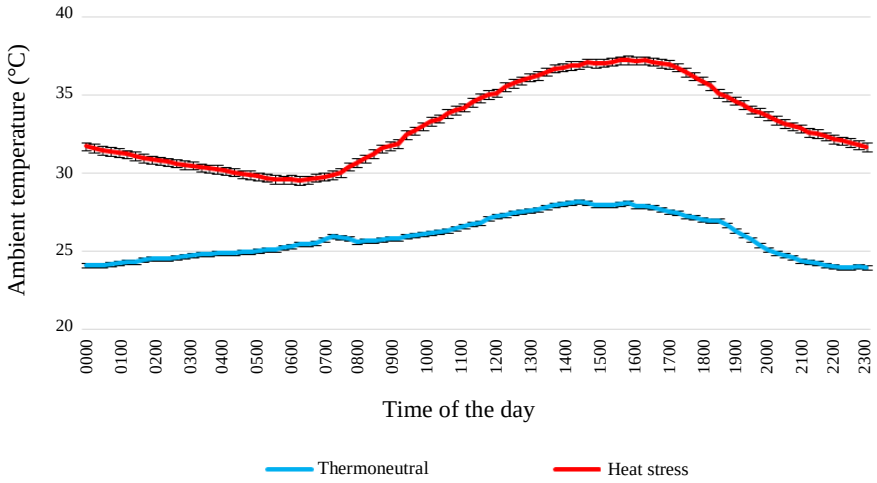

Fig. 1: Average ambient temperature inside the thermoneutral or heat stress room recorded at 15-min intervals

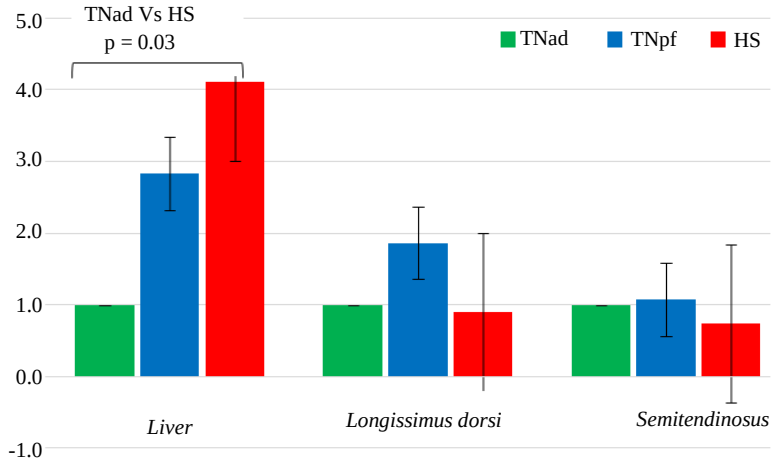

Fig. 2: Fold of increase-decrease on expression of mRNA for HSP70 analyzed in liver and *Longissimus dorsi* and *Semitendinosus* muscles of pigs under thermoneutral *ad libitum* (TNad), thermoneutral pair fed (TNpf) or heat stress (HS) conditions

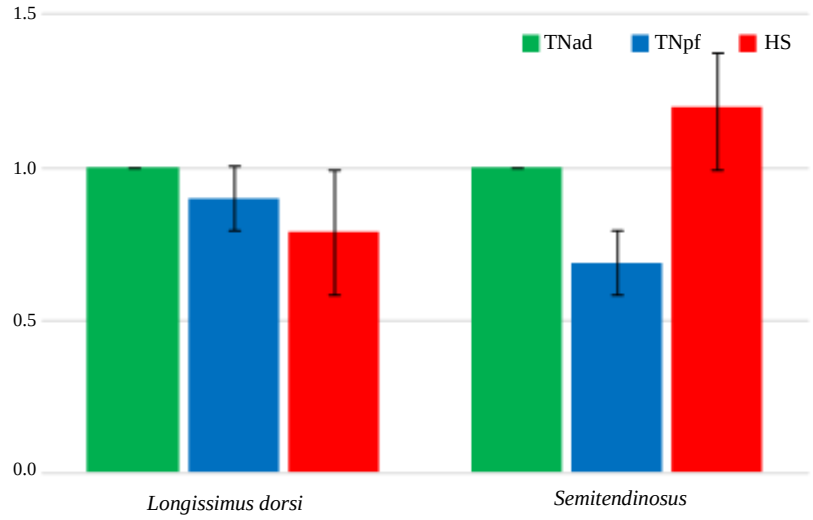

Fig. 3: Fold of increase-decrease on expression of mRNA for UCP3 analyzed in *longissimus* dorsi and *semitendinosus* muscles of pigs under thermoneutral *ad libitum* (TNad), thermoneutral pair fed (TNpf) or heat stress (HS) conditions

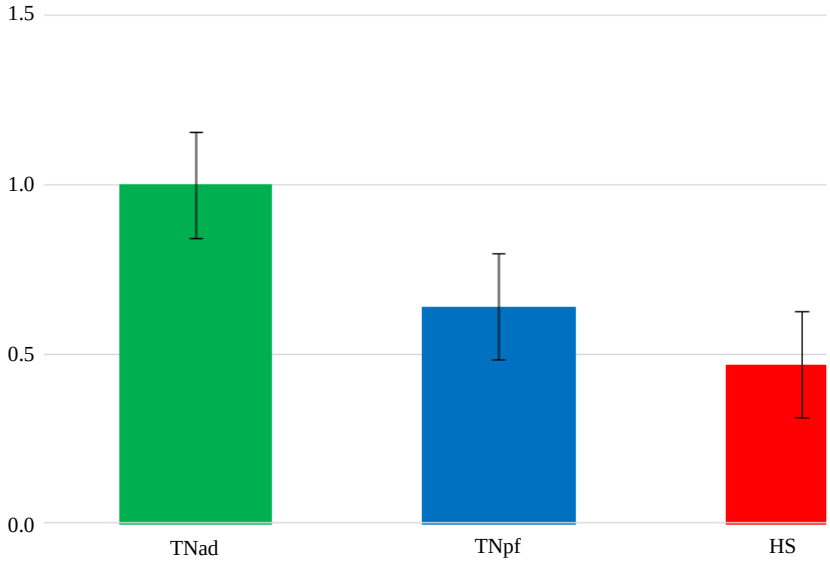

Fig. 4: Fold of increase-decrease on expression of mRNA for CYP3A analyzed in liver of pigs under thermoneutral *ad libitum* (TNad), thermoneutral pair fed (TNpf) or Heat Stress (HS) conditions

The mRNA expression of UCP3 in muscles (Fig. 3) was not affected in LD ($p \geq 0.855$) and ST muscle ($p=0.496$) by the exposure of pigs to high ambient temperature at similar feed intake (HS *vs.* TNpf). Similarly, there was no effect of the feed intake level on the UCP3 mRNA expression in LD ($p=0.880$) and ST ($p=0.648$) muscles of pigs housed under TN conditions (TNad *vs.* TNpf). Moreover, the UCP3 expression in the LD ($p=0.738$) and ST ($p=0.818$) muscles of HS pigs did not differ from that of TN pigs fed *ad libitum* (HS *vs.* TNad).

In liver, the mRNA expression of CYP3A (Fig. 4) was not affected by the exposure of pigs to high ambient temperature at similar feed intake (HS *vs.* TNpf; $p=0.574$), or by the feed intake level of pigs housed under TN conditions (TNad *vs.* TNpf; $p=0.411$). The expression of CYP3A in HS pigs did not differ from that of TN pigs fed *ad libitum*. In this study, we were no able to detect the expression of UCP3 in liver, or CYP3A in the LD or the ST muscles.

Discussion

The expression of HSP70, UCP3 and P450 in muscles and liver of pigs exposed to natural HS or TN conditions and different feed intake is part of a large data set obtained in the present experiment. Performance, mRNA expression for amino acid transporters and serum amino acids are published already (Morales *et al*., 2016).

The thermo-neutral zone for nursery-growing pigs is around 24°C (Huynh *et al*., 2005; Straw *et al*., 2006); at this temperature pigs can maintain normal physiological constants and show optimal growth performance. In the present experiment, housing temperature of TNad and TNpf pigs was around 26°C, which is close to thermo-neutral zone. In contrast, HS pigs were exposed to an average AT of 33°C, about 7°C above TN zone; moreover, HS pigs were exposed to AT as high as 37.2°C (11.2°C above TN zone). Thus, pigs housed in the HS room were exposed to HS conditions all the time. In addition, as previously reported (Cervantes *et al*., 2016; Cervantes *et al*., 2018), pigs exposed to HS conditions similar to those of the present experiment, reduced their feed intake, had increased BT, breath rate and lethargic state mainly during the afternoon when the highest temperature was recorded. These productive and physiological parameters alterations confirm that HS pigs were exposed to HS conditions.

The exposure of pigs to high AT increases their BT (Renaudeau *et al*., 2010; Pearce *et al*., 2013; Cervantes *et al*., 2018). According to Flanagan *et al.* (1995) hyperthermia results in a tissue-specific accumulation of HSP72, mostly in internal organs like liver. Heat shock proteins assist in the assembly, repair and refolding of damaged proteins under normal and stress conditions such as hyperthermia, energy depletion and increased ROS, among others (Kregel, 2002). Thus, an increase in HSP is a signal of cellular response to cope with those stressful conditions (Lanneau *et al*., 2010). Several reports show increased HSP70 expression in blood cells of cattle (Kumar *et al*., 2007; Gaughan *et al*., 2013; Bharati *et al*., 2017) and goats (Dangi *et al*., 2015) under chronic HS.

In the present study, the expression of HSP70 in liver of pigs after 21 d of exposure to HS increased 4-fold compared to TNad. This increased HSP70 expression in liver coincides with the 5-fold increase in HSP70 expression in liver and brain of HS tolerant goats during

the peak of a HS period, compared to HS susceptible goats reported by Rout *et al.* (2016). Also Nagayach *et al*. (2017) observed an increase in HSP70 expression in liver and heart of goats during the summer. In agreement, we recently demonstrated that chronic HS increases the expression of HSP90 in liver, LD and duodenum of pigs (Morales *et al*., 2014; Cervantes *et al*., 2016). However, in the present study there was no effect of HS or feed intake on the expression of HSP70 in LD and ST muscles. Flanagan *et al*. (1995) reported that hyperthermia did not affect the accumulation of HSP72 in the peripheral tissues like muscles of the limbs. Although Pearce *et al*. (2013) observed an increase in the HSP70 expression in the LD muscle of pigs at d1 of HS exposure, it decreased at d3 and d7. This response indicates that the increased HSP70 expression in muscle may occur during a short period of time, thus animals may start to acclimate to chronic HS conditions (Gaughan *et al*., 2013). Other studies with pigs (Morales *et al*., 2014), cattle (Bharati *et al*., 2017), goats (Dangi *et al*., 2015) and mice (Sareh *et al.*, 2011) showed a reduction in HSP70 expression after chronic or repeated exposure to heat stress. This partially explains the lack of effect on expression of HSP70 in both muscles analyzed in the present experiment. Larger expression of HSP70, according to Lacetera *et al*. (2006), can be associated to lower heat tolerance.

UCPs are a family of mitochondrial anion-carrier proteins that generate heat by dissipating the mitochondrial proton gradient, uncoupling respiration from ATP synthesis; this represents an important mechanism for generating heat in animals at rest (Palmieri, 1994; Dridi *et al*., 2004). UCP2 and UCP3 have been detected in muscles of pigs (Damon *et al*., 2000). UCP3 is expressed predominantly in skeletal muscle, heart and brown adipose tissue (Echtay, 2007; Ramsay and Richards, 2007) but not in liver (Schrauwen, 2002). Accordingly, in the present experiment, we detected the expression of UCP3 only in LD and ST muscles, but it was not detected in the liver of pigs. The action mechanism of UCP3 involves the transport of fatty acid anions (or peroxides) resulting from fatty acid oxidation into the mitochondrial matrix. Thus it appears that UCP3 protects mitochondria against oxidative damage by preventing the accumulation of oxidative species or ROS (Echtay, 2007; Cioffi *et al*., 2009; Busiello *et al*., 2015). Mitochondrial oxidative damage occurs by accumulation of protons in the inter-membrane space and increased ROS emission from the electron transfer chain (Korshunov *et al*., 1997), whereas UCP3 uncouples the respiratory chain reducing the accumulation of ROS.

The expression of UCP3 is modified under stress conditions such as hyperoxia (Flandin *et al*., 2005), fasting (Cioffi *et al*., 2009; Busiello *et al*., 2015) and even under reduced protein intake (Ramsay and Mitchell, 2008) because of its activity in fatty acid oxidation. Hence UCP3 could have an important role under HS conditions, when reduced feed intake and high mitochondrial production of ROS is usually observed (Baumgard and Rhoads, 2013; Slimen *et al*., 2015). Increased ROS production has been reported in several HS animals (Huang *et al*., 2015; Kikusato *et al*., 2016; Banh *et al*., 2016). Reduced expression of avUCP in muscule after acute HS exposition of chickens (Kikusato *et al*., 2016) and heat acclimation of myocytes (Salgado *et al*., 2017) has been reported. Mujahid *et al*. (2007) observed that 18 h of heat stress (34°C) stimulated the superoxide production in mitochondria, probably by down-regulation of avUCP in muscle tissue. These authors hypothesized that appropriate UCP expression could help to a better adaptation to HS. The expression of UCP3 in muscle increased after the chronic exposure of pigs to HS in response to, apparently, the increased mitochondrial ROS production resulting from the exposure to heat (Katsumata *et al*., 2004). In the present experiment, however, neither HS nor feed intake affected the expression of UCP3, probably because pigs became acclimated after being exposed to to high AT during 21 d. Further studies are necessary in order to achieve a better understanding of the activity and function of these proteins.

The cytochrome enzymes CYPs are heme proteins located in the membrane of mitochondria and endoplasmic reticulum of several tissues including liver that participate in the detoxification process of compounds such as drugs (Rasmussen and Zamaratskaia, 2014). CYP3A is the most important and most studied mammalian heme protein (Sevrioukova and Poulos, 2013; Shang *et al*., 2013). Stress is a critical player in the regulation of most CYPs that could modify their activity and gene expression (Daskalopoulos *et al*., 2012). Decreased activity in rats (Damanhouri, 2002) and liver expression of this enzyme in mid-lactation of dairy cows due to the HS exposure (McCracken *et al*., 2015) were reported. Acute exposure of mouse to 40-42°C increased the expression of CYP3A in liver and promoted hepatocyte proliferation, but the exposure to 44-46°C inhibited proliferation and promoted apoptosis of hepatocytes (Li *et al*., 2012). Altered electron transport by cytochrome P450 in mammary epithelial cell culture exposed to 42°C during 1 h was also reported (Kapila *et al*., 2016). In the present experiment, the expression of CYP3A was detected only in liver, which is in agreement with other authors (Shang *et al*., 2013; Nielsen *et al*., 2017). However, the lack of effect of chronic HS on the expression of CYP3A may support the hypothesis that these pigs were acclimated to HS after the 21 exposure to high AT (Horowitz, 2001; Renaudeau *et al*., 2010; Yu *et al*., 2010). In general, it is

likely that the cells and its cytochromes became adapted to HS, reducing the damages caused by the environmental conditions and explain the lack of effect of HS in CYP3A and UCP3 expression.

Conclusion

After a long exposure to HS, pigs increased their expression of the cytosolic protein HSP70 in liver, probably in order to maintain their proteins integrity. But the expression of mitochondrial proteins UCP3 and CYP3A in liver and muscles were unaffected by HS or feed intake. These results suggest that HS pigs became acclimated to HS after the exposure to high AT for 21 d. Nevertheless, factors other than mitochondrial ROS production, increased BT and lower feed intake appear to be responsible for the poorer performance observed in pigs acclimated to HS.

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Author's contribution

V. Montesinos-Cruz: Carried out field and laboratory work, analyzed and interpreted the data for the manuscript, wrote a pre-manuscript.

M. Cota: Collaborated in fieldwork and participated in discussion of results.

L. Buenabad: Collaborated in laboratory work and data analysis.

M. Cervantes: Designed the experiment, advised field work and proofread the manuscript.

A. Morales: Designed and supervised the experiment and laboratory work, wrote the manuscript.

Ethics

The authors confirm that the present article is original and no ethical issues are concerned with it.

Conflict of Interest

The authors declare that they have no conflict of interest regarding the publication of this paper.

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