EFFECT OF SEX AND GENOTYPE ON STRESS BIOMARKERS IN PIGS

M. Oliván[1*], J. González[2], A. Bassols[3], N. Panella-Riera[2], R. Carreras[2], I. Vega-Naredo[4], E. Mainau[3], L. Arroyo[3], R. Peña[3], Y. Potes[4], A. Coto-Montes[4], K. Hollung[5], X. Manteca[3], A. Velarde[2]

[1]SERIDA, Apdo 13, 33300 Villaviciosa, Asturias, Spain.
[2]IRTA, Finca Camps i Armet s/n, 17121 Monells, Spain.
[3]Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Spain.
[4]Universidad de Oviedo, C/ Julián Clavería s/n, 33006 Oviedo, Asturias, Spain.
[5] Nofima, AS, PO BOX 210, N-1431 Aas, Norway.
*mcolivan@serida.org

Abstract – The aim of this work was to identify physiological, biochemical and proteomic biomarkers to assess the individual response to slaughter stress of pigs of different sex (male "M"/female "F") and halothane genotype (NN/Nn). Forty-eight pigs were reared simulating commercial conditions and distributed in four treatments (M-NN, M-Nn, F-NN, F-Nn). At slaughter, blood samples were taken for biochemical analysis. Carcass and meat quality traits (pH, skin lesions, electrical conductivity, color, drip loss, toughness) were measured and muscle samples were taken for SDS-PAGE proteomic analysis. There was a significant effect of sex and genotype on animals' susceptibility to stress. These differences could be monitored by using some physiological, biochemical and proteomic biomarkers related to muscle fiber composition and oxidative stress.

I. INTRODUCTION

The welfare status of farm animals is becoming an important aspect of overall food quality from the consumer point of view. However, animal welfare assessment is not a simple issue and requires a multi-criteria approach.

This study was performed within the ANEMOMA project, which addresses the demand for improved knowledge on animal welfare in pigs, its relationship with meat quality and the identification of novel animal-based biomarkers of stress.

Previous studies have shown that sex (male "M"/female "F") and halothane gene mutation (NN/Nn) may influence the *post mortem* process of muscle-to-meat conversion [1, 2]. Moreover, these factors could be influenced by the individual animal susceptibility to *ante* and *peri mortem* stress.

The objective of this work was to identify physiological, biochemical and proteomic biomarkers to assess the individual response to slaughter stress of pigs of different sex (M/F) and halothane genotype (NN/Nn).

II. MATERIALS AND METHODS

Animals and Samples

Forty-eight [(Large White x Landrace) x Pietrain] pigs were reared in the experimental farm of IRTA in Monells (Girona, Spain), simulating commercial conditions. Animals were distributed in groups of 6 pigs per pen, corresponding to four treatments (M-NN, M-Nn, F-NN, F-Nn) in duplicate. There were two slaughter batches, in two consecutive weeks, including 24 animals per day. In the slaughter day they were transported using a lorry from the farm to the experimental abattoir in IRTA Monells, lasting for less than 10 minutes. Four pigs from two different groups were transported per trip without mixing groups, they were located in the lairage pens maintaining the groups, and after 30 minutes they were slaughtered. At slaughter, blood samples were taken for biochemical analysis. Serum was obtained after centrifugation at 3000 rpm 10 min. Metabolites analyzed were glucose, lactate, urea, creatinine, total protein, lipid metabolism markers (triglycerides (TGs), total cholesterol (Chol), HDL-cholesterol, LDL-cholesterol, non-esterified fatty acids (NEFAs) and beta-hydroxybutyrate (BHB)); acute phase proteins (haptoglobin (Hp), C-reactive protein (CRP) and Pig-MAP); skeletal muscle marker (creatine kinase (CK)); redox marker (glutathione peroxidase (GPx)); and cortisol. All parameters were determined by spectrophotometric techniques in the analyzer Olympus AU400, with the exception of Pig-MAP and cortisol, which were determined by ELISA.

The left side of each carcass was used to assess meat quality. Then, pH was measured at 45 minutes (pH45) and at 24 hours (pH24) *post mortem* on the *Semimembranosus* (*SM*) and *Longissimus dorsi* (*LD*) muscles, using a Crison portable pHmeter equipped with a xerolyt electrode. Also, electrical conductivity (EC) was measured at 45 minutes *post mortem* on the *SM* and *LD* using a Pork Quality Meter (PQM-I, INTEK Aichach).

The skin lesions in each animal were assessed using the Welfare Quality® protocol [3], considering 5 regions on the carcass and values of 0 (<2 lesions in all regions), 1 (2-10) and 2 (>10).

Meat color measurements (L*, a*, b*) were taken at 24 h *post mortem* on the exposed cut surface of the muscle at the last rib level, after 15 minutes blooming, using a Colorimeter Minolta CR-400 in the CIELAB space.

Meat drip loss (% exudates) was determined by duplicate on 25 mm diameter fresh samples taken from the *LD* muscle at 24 h *post mortem* and placed on a special container (Meat juice collector, Sarstedt), according to the method of Rasmussen and Andersson [4], with small modifications.

Meat samples (20 g) were taken from the *LD* muscle immediately after slaughter for analysis of electrophoretic protein profile of sarcoplasmic extracts by SDS-PAGE. Proteins were extracted and quantified following the method described by Jia *et al.* [5]. Stained gel images were captured using the UMAX ImageScanner (Amersham Biosciences) and the protein bands were quantified with the ImageMaster software. The protein spots of interest were manually excised from gels and analyzed with a 4800 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (ABSciex).

Instrumental texture was determined in *LD* samples by using the Warner Bratzler test, obtaining the shear force. Samples were vacuum packaged at 24 hours *post mortem* and stored at 4ºC, and they were frozen (-20ºC) at 1, 3 and 5 days *post mortem* to allow muscle tenderization. Each sample was thawed overnight, cooked in an oven until a core temperature of 71ºC, and then 5 subsamples were obtained by using a perforating punch. They were individually analyzed in the texturometer and the mean value of each sample was calculated.

Statistical Analysis

The effect of sex (M/F) and genotype (NN/Nn) on carcass and meat quality traits, blood biochemical and muscle proteomic variables was analyzed by Analysis of Variance (ANOVA) using the General Linear Model (GLM) procedure of SPSS (*v* 15.0 2006, SPSS Inc). The model included sex, genotype and its interaction as fixed factors and slaughter day as random factor.

Also, multivariate analysis (PCA) was performed with XLStat (XLStat, 2013) in order to study the complex relationships between meat quality and physiological, biochemical and proteomic variables.

III. RESULTS AND DISCUSSION

Carcass and meat quality

Females showed a lower muscle pH (Table 1), being the difference significant at 45 min *post mortem* in *LD* muscle (6.23 *vs* 6.39, females *vs* males, $p<0.01$) and at 24 h *post mortem* in *SM* muscle (5.45 *vs* 5.50, females *vs* males, $p<0.05$). Meat from females showed also significantly higher values of EC in the *SM* than males (7.61 *vs* 5.69, $p<0.01$).

Table 1 Effect of sex (S) and genotype (G) and its interaction (SxG) on carcass and meat quality traits.

	Effect		
	S	G	SxG
Temperature (ºC)	NS	NS	NS
Skin lesions	NS	NS	NS
pH45*LD*	**	***	NS
pH45*SM*	NS	***	NS
pH24*LD*	NS	NS	NS
pH24*SM*	*	NS	NS
EC-*LD* (mS)	NS	*	NS
EC-*SM* (mS)	**	*	NS
Drip loss (%)	NS	***	NS
L*	NS	NS	NS
a*	NS	**	*
b*	NS	NS	*
Shear force (WB,kg) 1 day	NS	*	NS
Shear force (WB,kg) 3 days	NS	**	NS
Shear force (WB,kg) 5 days	NS	***	NS

Furthermore, it seems that the *post mortem* pH decline was faster in animals heterozygous for the halothane mutation (Nn), that showed significantly ($p<0.001$) lower pH at 45 min in both studied muscles. Also, Nn animals produced more exudative (higher EC and drip loss) and tougher meat (higher WB, reduced meat tenderization) (Table 1). Regarding instrumental color, the variable L* did not show any significant difference for the analyzed factors. Nevertheless, there was a significant

interaction Sex x Genotype (SxG) for some meat color traits (a*, b*).

Blood biochemical variables

Sex affected significantly some blood metabolites. In general, females showed higher levels than males of glucose (358.5 *vs* 308.4 mg/dL, $p<0.05$), urea (35.30 *vs* 26.20 mg/dL, $p<0.001$), CRP (11.49 *vs* 7.20 µg/mL, $p<0.05$), Pig-MAP (0.84 *vs* 0.60 mg/mL, $p<0.01$) and GPx (11600 *vs* 8589 U/L, $p<0.01$) and lower of lactate (8.93 *vs* 10.49 mmol/L, $p<0.05$). When looking to differences between groups (Figure 1), it is worthwhile to mention that increased urea concentration in females was consistent in both NN and Nn groups, suggesting a faster catabolism of proteins, maybe associated to higher stress susceptibility.

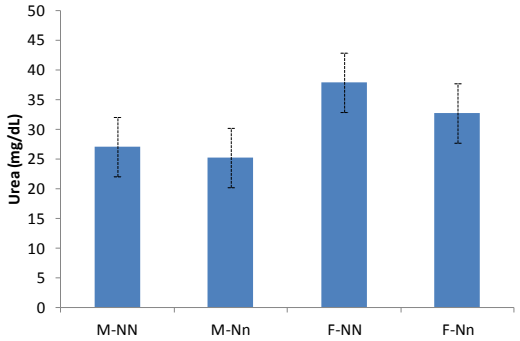

Figure 1. Urea blood levels in the four studied treatments (M: male, F: female, NN: homozygous, Nn: heterozygous).

Genotype affected significantly CK (3696 *vs* 1909 U/L, Nn *vs* NN, $p<0.001$), creatinine (2.04 *vs* 1.94 mg/dL, Nn *vs* NN, $p<0.05$), CRP (11.20 vs 4.79 µg/mL, Nn *vs* NN, $p<0.05$), Pig-MAP (0.65 *vs* 0.79, Nn *vs* NN, $p<0.05$) and GPx (11385 *vs* 8763 U/L, NN *vs* Nn, $p<0.01$).

Heterozygous (Nn) showing higher CK activity (Figure 2) indicates a higher susceptibility to muscular lesions, and higher CRP concentration indicates higher stress level and subsequent inflammation. On the other side, the higher GPx activity in homozygous (NN), especially in females (there was significant SxG interaction, $p<0.01$) suggests more potent antioxidant defenses probably due to estrogen influence (Figure 3).

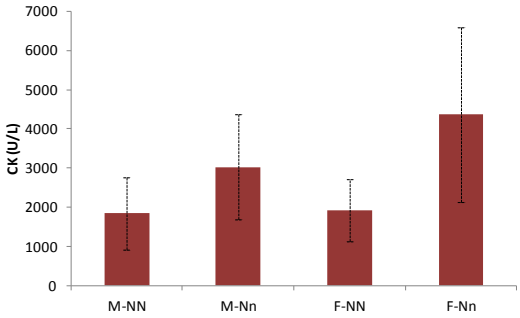

Figure 2. CK blood levels in the four studied treatments (M: male, F: female, NN: homozygous, Nn: heterozygous).

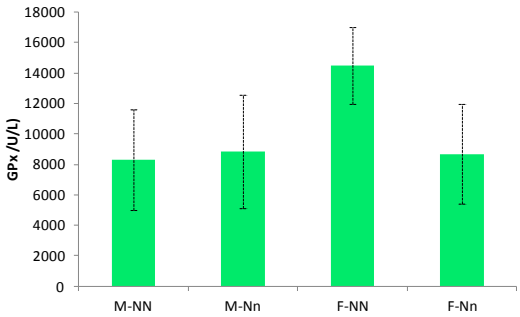

Figure 3. GPx blood levels in the four studied treatments (M: male, F: female, NN: homozygous, Nn: heterozygous).

Muscle proteins

A total of 26 protein bands were differentiated by SDS-PAGE gels in this study (Figure 4). Table 2 shows noticeable bands for which there were significant differences between treatments

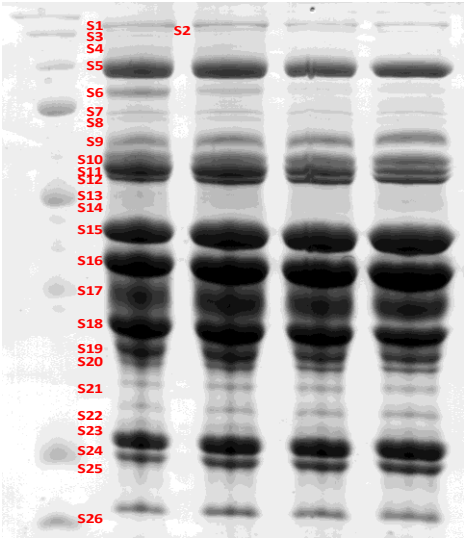

Figure 4. SDS-PAGE gel image of sarcoplasmic extracts of *LD* muscle. Bands names are denoted by S (sarcoplasmic protein) followed by a number.

Table 2 Effect of sex (S) and genotype (G) and its interaction (SxG) on noticeable proteins of the muscle extracts

	Effect		
	S	G	SxG
S2-Myosin-binding protein C fast type	*	*	NS
S6-Muscle 6-phosphofructokinase	**	NS	NS
S9-Albumin, partial	NS	NS	**
S18-Glyceraldehyde-3-phosphate dehydrogenase "GAPDH"	NS	**	NS
S23-Beta-enolase	NS	*	NS
S24-Carbonic anhydrase 3	NS	*	NS

Sex affected significantly the presence of S2 (myosin-binding protein C fast type, $p<0.05$), that showed higher values in the muscle extracts from females, and S6 (muscle-6-phosphofructokinase, $p<0.01$), with lower values in females. These results reflect the effect of gender on the fiber composition of skeletal muscle and on the *post mortem* glycolytic pathway.

Genotype affected the presence of four proteins, with lower S2 (myosin-binding protein C fast type, $p<0.05$), S18 (glyceraldehyde-3-phosphate dehydrogenase "GAPDH", $p<0.01$) and S24 (carbonic anhydrase, $p<0.05$) and higher S23 (beta-enolase, $p<0.05$) in Nn genotype, which could imply differences in fiber composition and the resulting *post mortem* evolution of oxidative stress.

Multivariate analysis

PC1 and PC2 explained 39% of the variability of data (Figure 5).

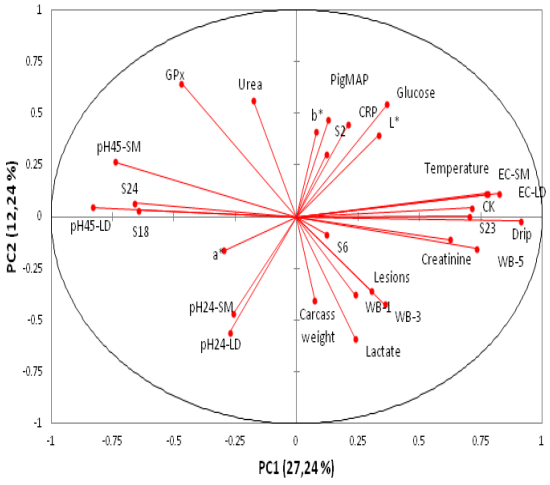

Figure 5. PCA biplot of main meat quality traits and stress biomarkers.

PC1 distinguished in the right side exudative (higher drip and EC) and tougher meat (higher WB), obtained from Nn animals, that showed higher levels of creatinine and CK in blood and beta-enolase (S23) in muscle. Also, higher skin lesions and *post mortem* carcass temperature characterized these samples, which seem to indicate higher *peri mortem* stress. In the opposite side of PC1, meat from NN genotype showed slower rate of pH decline (higher pH-45) and higher muscle levels of GAPDH (S18) and carbonic anhydrase (S24).

PC2 distinguished in the positive side meat from females, with higher blood levels of GPx, urea, Pig-MAP and CRP at slaughter, variables indicating an inflammatory and antioxidant response to stress.

IV. CONCLUSION

The results of this study showed that sex and genotype affected stress biomarkers in pigs. In general, females and animals heterozygous for the halothane mutation (Nn) showed higher susceptibility to stress. These differences could be monitored by using some physiological, biochemical and proteomic biomarkers related to muscle fiber composition and oxidative stress.

ACKNOWLEDGEMENTS

Work supported by project AGL2011-30598-C03 (Ministry of Economy and Competitiveness of Spain).

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