This document is a postprint version of an article published in Meat Science © Elsevier after peer review. To access the final edited and published work see https://doi.org/10.1016/j.meatsci.2018.03.018

TITLE:

Effect of sex and RYR1 gene mutation on the muscle proteomic profile and main physiological biomarkers in pigs at slaughter

AUTHORS' NAMES AND AFFILIATIONS:

Mamen Oliván [a,*], Joel González [b], Anna Bassols [c], Fernando Díaz [a], Ricard Carreras [b], Eva Mainau [b], Laura Arroyo [c], Raquel Peña [c], Yaiza Potes [d], Ana Coto-Montes [d], Kristin Hollung [e], Antonio Velarde [b]

[a] SERIDA, Apdo 13, 33300 Villaviciosa, Asturias, Spain.
[b] IRTA, Finca Camps i Armet s/n, 17121 Monells, Spain.
[c] Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Spain.
[d] Universidad de Oviedo, C/ Julián Clavería s/n, 33006 Oviedo, Asturias, Spain.
[e] Nofima, AS, PO BOX 210, N-1431 Aas, Norway.

Preliminary data from this work were partially presented at the International Congress of Meat Science and Technology (ICOMST) held at Punta del Este, Uruguay, on August 2014.

*Corresponding author:

E-mail: mcolivan@serida.org (M. Oliván)

Postal address: SERIDA, Apdo 13, 33300 Villaviciosa, Asturias, Spain

ABSTRACT

Gender and RYR1 gene mutation might have an effect on the muscle metabolic characteristics and on the animal's stress at slaughter, which could influence the process of muscle-to-meat conversion. Forty-eight pigs were distributed in a design including two factors: sex (male/female) and RYR1 genotype (NN/Nn). At slaughter, physiological blood biomarkers and muscle proteome were analysed and carcass and meat quality traits were registered. Females had higher serum levels of glucose, urea, C-reactive protein "CRP", Pig-MAP and glutation-peroxidase "GPx" and lower levels of lactate, showed faster muscle pH decline and higher meat exudation. RYR1 mutation increased serum creatinine, creatine kinase and CRP and decreased GPx. The proteomic study highlighted significant effects of gender and RYR1 genotype on proteins related to fibre composition, antioxidant defense and *post mortem* glycolytic pathway, which correlate to differences of meat quality. This study provides interesting information on muscle biomarkers of the ultimate meat quality that are modulated by the animal's individual susceptibility to stress at slaughter.

Key words: sex, RYR1, pig, biomarker, proteomics, stress

1. Introduction

Meat quality should be considered as a multifactorial trait, including technological, nutritional, sensory, safety and ethical aspects, and is affected by several factors, such as the genetic animal type, the particularities of the production system, the physiological response of the animals to the *ante mortem* treatment and the *post mortem* conditioning of the carcasses, among others.

The effect of gender on the pig carcass development has been described in the literature. In general, males present less accumulation of fat tissues (Gispert et al., 2010), but there is still controversy about the effect of gender on the incidence of pale, soft and exudative (PSE) meat, with some studies showing significant effects (Cisneros, Ellis, McKeith, McCaw, & Fernando, 1996; Channon, Kerr, & Walker, 2004) whereas others did not (Blanchard, Warkup, Ellis, Willis, & Avery, 1999; Channon, Payne, & Warner, 2000). These differences between experiments could be due in part to different *ante mortem* handling conditions, which may influence the animal stress reactions at slaughter and could have consequences on the ultimate meat quality (Boler et al., 2010; Hambrecht et al., 2005a,b; Terlouw and Rybarczyk, 2008; D'Eath et al., 2010).

The most usual crossbreds used in Spain includes the Pietrain sire line, presenting in most cases heterozygosity (Nn) to the mutated RYR1 gene. Mutations in the RYR1 gene have been related to higher susceptibility to stressful conditions and to the induction of malignant hyperthermia in pigs (Fujii et al., 1991), with detrimental effects on meat quality (Fàbrega et al., 2004).

Then, pigs of different sex and genetic types may respond differently to pre-slaughter handling, which may affect the *post mortem* process of muscle-to-meat conversion. This process implies complex biochemical mechanisms that are to a large extent dependent

on the genetic background, the tissue physiological milieu and the animal's perception of danger or fear during the slaughter procedure.

To date, there is still no precise definition of animal stress, probably due to the complexity of different physical and psychological stressful situations, although it can be described as "the physiological, behavioural and psychological state of the animal when confronted with, from the animal's point of view, a potentially threatening situation" (Terlouw, 2005).

Furthermore, it is important to note that the stress level of the animal depends indirectly on the situation and directly on the animal's evaluation of the situation (Terlouw, 2005). For this reason, meat scientists show increasing interest in the identification of animal-based biomarkers that could be indicators of stress at slaughter and even that could be used as indirect predictors of the ultimate meat quality. In this field, proteomics is a promising tool, although its application is still in its infancy and very few studies have focused on stress-dependent muscle proteome changes (Franco et al., 2015; Oliván et al., 2016). The objective of this work was to investigate the effect of gender (Male/Female) and RYR1 genotype (NN/Nn) on physiological, biochemical and proteomic variables detected in the carcass that might influence the process of meat quality acquisition and reflect animal's susceptibility to stress at slaughter.

2. Materials and Methods

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of IRTA (Monells, Spain). The care and use of animals were performed in accordance with the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes (EU, 2010).

237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295

94 *2.1. Animals and Management procedures*

95 Forty-eight crossbred pigs ([Large White x Landrace] sows sired with Pietrain boars)
96 were randomly selected at a commercial farm and assigned to four groups of 12 pigs
97 each one. Each group either consisted of NN females, NN entire males, Nn females and
98 Nn entire males. The RYR1 genotype of the pigs was determined from a hair sample
99 using PCR (polymerase chain reaction) amplification and digestion with restriction
100 enzymes as described by Fujii et al. (1991) when pigs aged 5 weeks.
101 At 9 weeks of age, pigs were transported from the commercial farm to the experimental
102 facilities of IRTA and housed separately by treatment (sex x genotype) in 8 pens (6 pigs
103 per pen), that is, two replicas per treatment. Pigs were kept in pens (5 x 2.7 m) on fully
104 slatted floor under natural light conditions and at a constant environmental temperature
105 of 22 ± 3°C. Each pen was provided with one steel drinker bowl (15 x 16 cm) connected
106 to a nipple and with a concrete feeder (58 x 34 cm) with four feeding places. Pigs had
107 water and feed *ad libitum*. Pigs were inspected daily and no health problems were
108 observed during the experimental period. At an average weight of 111.4 ±10.5 kg the
109 pigs were fasted for 8 h before being transported to the experimental slaughterhouse of
110 IRTA (1.2 km trip), without mixing groups. Animals were gently handled during
111 transport and at the slaughterhouse to avoid additional stress. There were two slaughter
112 batches, in two consecutive weeks, including 24 animals per day. Slaughtering started
113 30 min after the animals arrived at the lairage pens and lasted for 3 h. Pigs were stunned
114 by exposure to 90 % of carbon dioxide (CO_2) by volume in atmospheric air during 3
115 min and exsanguinated afterwards.
116

117 *2.2. Blood collection*

At the slaughterhouse, blood samples were collected at exsanguination from each pig in 10-mL tubes without anticoagulant. Serum were obtained by centrifugation at 2000× g for 10 min and immediately frozen at −80 °C until analysis.

2.3. Biochemical and physiological parameters

Metabolites analyzed were:

- Markers for glucose utilization: glucose (Hexokinase method, Olympus System Reagent OSR), lactate (Enzymatic method LOD -Lactate Oxidase-, Olympus System Reagent OSR).
- Markers of nitrogen metabolism: creatinine (Jaffé method, Olympus System Reagent OSR), urea (GLDH method, Olympus System Reagent OSR), total proteins (Biuret method, Olympus System Reagent OSR).
- Lipid metabolism markers: triglycerides (GPO-PAP method, Olympus System Reagent OSR), total cholesterol (CHOP-PAP method, Olympus System Reagent OSR), HDL-cholesterol (HDL-chol, Immunoinhibition method, Olympus System Reagent OSR), LDL-cholesterol (LDL-chol, Selective protection method, Olympus System Reagent OSR), non-esterified fatty acids (NEFAs, NEFA-C reagent, Wako Chemicals) and 3-hydroxybutyrate (BHB, Ranbut reagent, Randox Laboratories, Ltd).
- Acute phase proteins “APPs” as inflammatory markers: haptoglobin (Phase Haptoglobin, Tridelta Ltd), C-reactive protein (CRP, immunoturbidimetric method, Olympus Systems Reagent) and Pig-MAP (ELISA, PigChamp ProEuropa).
- Skeletal muscle marker: creatine kinase (CK, IFCC method, Olympus System Reagent OSR).
- Oxidative stress marker: glutathione peroxidase GPx (Cumene Hydroperoxyde method, Ransel, Randox Laboratories Ltd).

- Stress hormone: cortisol (ELISA, DRG Diagnostics, Germany).

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.

2.4. Carcass measurements and meat sampling

The skin lesions in each pig were assessed using the Welfare Quality® protocol (Dalmau, Temple, Rodríguez, Llonch, & Velarde, 2009) considering 5 regions (ears, front, middle, hind-quarters and legs) in one side of the carcass after scalding. Values of 0 (<2 lesions in all regions), 1 (2-10 lesions in at least one region) and 2 (>10 lesions in at least one region) were used by a trained observer at the slaughter line.

The left side of each carcass was used to assess meat quality. Muscle pH was measured at 45 min (pH45) and at 24 h (pH24) *post mortem* on the *longissimus thoracis* (LT) muscle, using a Crison (Hach Lange S.L.U., Spain) portable meter equipped with a xerolyt electrode. Electrical conductivity (EC) was also measured at 24 h *post mortem* on the same location using a Pork Quality Meter (PQM-I, INTEK Aichach, Germany).

Meat samples (20 g) were taken after slaughter (15-20 min *post mortem*) from the LT muscle of each pig at the last rib level for analysis of electrophoretic protein profile of sarcoplasmic extracts by SDS-PAGE. These muscle samples were immediately frozen in liquid nitrogen and stored at -80ºC until analyzed.

Meat color was determined using a colorimeter Minolta CR-400 (Konica Minolta Holdings, Inc, Japan) measuring in the CIELAB space (L*, a*, b*), at 24 h *post mortem* on the exposed cut surface of the LT muscle (last rib) after 15 min blooming.

Meat drip loss (% exudates) was determined by duplicate on 25 mm diameter fresh samples taken from the LT muscle at 24 h *post mortem,* and placed on a special

container (Meat juice collector, Sarstedt, Nümbrecht, Germany) during 24 hours at 4ºC, obtaining the drip loss percentage by gravimetry, according to the method described by Rasmussen and Andersson (1996).

Instrumental texture was determined in LT samples by using the Warner Bratzler test, following the procedures described in Ampuero-Kragten and Gil (2015). Samples were vacuum packaged at 24 h *post mortem* and stored at 4ºC, and they were frozen (-20ºC) after 1, 3 and 5 d aging to allow muscle tenderization. Each sample was thawed overnight at 4ºC, cooked in an oven until a core temperature of 71ºC, and then 5 subsamples were obtained by using a perforating punch. These subsamples were individually analyzed for instrumental toughness (maximum shear force, in kg) with the TA.XT plus Texture Analyzer (Stable Microsystems, Haslemere, UK) and the mean value for each animal was calculated.

2.5. Sarcoplasmic Protein Extraction and Electrophoresis

The sarcoplasmic protein fraction, which contains most of the enzymes of the glycolytic pathway and other metabolic proteins (Hollung et al., 2007), were extracted from each individual muscle sample (one per animal), taken immediately after slaughter, and quantified following the method described by Jia et al. (2009). A total of 600 mg of muscle tissue was dissected and homogenized in 2 mL of Tris-EDTA-Sucrose “TES” buffer (10 mM Tris [pH7.6], 1 mM EDTA, and 0.25 M sucrose), using a Polytron PT1200 E (Kinematica Inc., Luzern, Switzerland) three times for 15 s at maximum speed. The homogenate was centrifuged (30 min at 8,800 x *g*) at 4°C to remove TES-insoluble proteins. Protein concentrations were measured with a commercial kit at 760 nm (RC DC Protein Assay, Bio-Rad Laboratories, Hercules, CA) in a

473
474
475 192 spectrophotometer Lambda 35 UV/VIS (Perkin Elmer, Massachusetts, USA) using
476
477 193 bovine serum albumin as standard.
478
479 194 Then, 120µg of proteins were denatured by mixing with sample buffer (62.5 mM
480
481 195 Tris/HCl pH 6.8, 2% SDS, 20% glycerol, 5% mercaptoethanol, 0.025% of bromophenol
482
483 196 blue) and heated at 95ºC for 5 min, and loaded to 1mm dual vertical slab gels (Xi
484
485 197 Protean II, Bio-Rad Laboratories Inc., CA, USA) for one-dimensional sodium
486
487 198 dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE), according to the
488
489 199 procedure described by Sierra et al. (2012). The resolving gel contained 11% and the
490
491 200 stacking gel 4% of 30% (wt/vol) acrylamide: bisacrylamide and a mixture of Tris/HCl
492
493 201 (375 mM) pH 8.8, milli-Q water, SDS 10% (wt/vol), ammonium persulphate 10%
494
495 202 (wt/vol), and 0.1% TEMED. Pre-stained molecular weight standards (Precision Plus
496
497 203 Protein All Blue Standards, Bio-Rad Laboratories Inc., Hercules, CA) were also run on
498
499 204 each gel to determine protein band molecular weights. Gels (20 cm x 20 cm size) were
500
501 205 run at 80 V for 2 h, 160 V for 2 h, 250V for 10 h and 500 V for 20 min (Universal
502
503 206 PowerPack 500, Bio-Rad), stained in a mixture of 30% (vol/vol) methanol, 10%
504
505 207 (vol/vol) acetic acid and 0.01% (wt/vol) Coomassie Brilliant Blue R-250 and destained
506
507 208 using a mixture of 40% (vol/vol) methanol and 10% (vol/vol) acetic acid.
508
509 209 Three gels were produced per muscle sample and the mean value was calculated for
510
511 210 each animal with image analysis techniques.
512
513 211
514
515 212 *2.6. Image Analysis and protein identification by peptide mass fingerprint*
516
517 213 Stained gel images were captured using the UMAX ImageScanner (Amersham
518
519 214 Biosciences). SDS-PAGE densitometry analysis and band quantitation were carried out
520
521 215 using the ImageQuant TL software by means of its 1D gel analysis tool (version 7.0,
522
523 216 GEHealthcare, Buckinghamshire, UK). To account for slight variations in protein
524

525
526
527
528
529
530
531

loading, the density protein bands was expressed as relative abundance (normalized volume) and expressed in arbitrary units.

Protein bands were manually excised from gels and sent for identification to the proteomics laboratory of Inbiotec S.L. (León, Spain). The proteins were digested following the method of Havlis et al. (2003) and processed for further analysis as indicated by Jami, Barreiro, García-Estrada, & Martín (2010). The samples were analyzed with a 4800 Proteomics Analyzer matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF/TOF) mass spectrometer (ABSciex, MA, USA). A 4700 proteomics analyzer calibration mixture (Cal Mix 5, ABSciex) was used as external calibration. All MS spectra were internally calibrated using peptides from the trypsin digestion. The analysis by MALDI-TOF/TOF mass spectrometry produced peptide mass fingerprints, and the peptides observed (up to 65 peptides per spot) were collected and represented as a list of monoisotopic molecular weights with a signal to noise (S/N) ratio greater than 20 using the 4000 Series Explorer v3.5.3 software (ABSciex). All known contaminant ions (trypsin- and keratin- derived peptides) were excluded for later MS/MS analysis. Hence, from each MS spectra, the 10 most intensive precursors with a S/N greater than 20 were selected for MS/MS analyses with CID (atmospheric gas was used) in 2-kV ion reflector mode and precursor mass windows of ±7 Da. The default calibration was optimized for the MS/MS spectra. For protein identification, Mascot Generic Files combining MS and MS/MS spectra were automatically created and used to interrogate a non-redundant protein database using a local license of Mascot v 2.2 from Matrix Science through the Global Protein Server v 3.6 (ABSciex). The search parameters for peptide mass fingerprints and tandem MS spectra obtained were set as follows: i) NCBInr (2012.09.13) sequence databases were used; ii) taxonomy: All entries (20363435 sequences, 6986060206 residues); iii) fixed and variable

modifications were considered (Cys as S carbamidomethyl derivative and Met as oxidized methionine); iv) one missed cleavage site was allowed; v) precursor tolerance was 100 parts per million and MS/MS fragment tolerance was 0.3 Da; vi) peptide charge: 1+; and vii) the algorithm was set to use trypsin as the enzyme. Protein candidates produced by this combined peptide mass fingerprinting/tandem MS search were considered valid when the global Mascot score was greater than 85 with a significance level of $P < 0.05$.

2.7. 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, Chicago, USA). The model included sex, genotype and its interaction as fixed factors and slaughter day (batch) as random factor. When the interaction was significant, the differences between the four treatments (M-NN, M-Nn, F-NN, F-Nn) were analysed by the Tukey post-hoc test. The *post mortem* evolution of meat toughness (Warner Bratzler maximum shear force) was analysed by GLM including sex, genotype, aging time and their interactions as fixed factors and animal as random factor. Bivariate correlations were calculated using Pearson's correlation coefficient.

Furthermore, multivariate analysis (PCA) was performed in order to study the relationships between meat quality and physiological, biochemical and proteomic variables obtained for every animal studied, by using XLStat software (XLStat 2013, Addinsoft Inc, Paris, France). The Kaiser-Meyer-Olkin test was performed in order to measure sampling adequacy for each variable in the model, and only variables with

KMO over 0.6 were selected. The overall KMO measure of the performed PCA was 0.725.

3. Results and discussion

3.1. Carcass and meat quality

Female pigs showed lower muscle pH than entire males (Table 1), with significant differences at 45 min *post mortem* (P = 0.002). This agrees with D'Souza and Mullan (2002) and D'Eath et al. (2010), who found lower pH (P = 0.006) in the loin muscle of females compared with castrated pigs. These differences may be in part due to physiological and metabolic differences in the cell response, indicating in some extent higher susceptibility of females to stress at slaughter. This is a controversial issue, as it has been postulated that entire male pigs are more susceptible to stress, because they show more aggressive behavior than females and castrates (Fàbrega et al., 2010), while in cattle Tarrant (1990) showed that females and young animals are more susceptible to stress compared to males and older animals.

When looking to the effect of genotype, we found that the *post mortem* muscle pH decline was faster in animals heterozygous for the RYR1 mutation (Nn), that showed significantly (P < 0.001) lower pH at 45 min *post mortem*, while the ultimate pH did not differ between genotypes (Table 1), so the pH amplitude (45 min - 24 h) was lower in the Nn group (0.76 vs 1.03 for Nn and NN, respectively, P < 0.05).

Furthermore, Nn animals produced meat with higher values of electrical conductivity (EC) (P < 0.05) and also higher drip loss (P < 0.001). Thus, the pH amplitude correlated negatively with EC (r=-0.702, P < 0.001) and drip loss (r=-0.726, P < 0.001) and EC and drip loss showed a positive and significant correlation (r= 0.858, P < 0.001). These differences seem to indicate *post mortem* muscle metabolic differences due to the RYR1

709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767

291 mutation but also might be modulated by a higher susceptibility to stress at slaughter in
292 Nn pigs, which have more intense reaction to stress than NN animals (Roberts et al.,
293 1998). This effect could produce higher leakage of calcium to the cytoplasm and the
294 accompanied calcium related effects (e.g. muscle contraction, stimulation of the muscle
295 metabolism) resulting in a rapid reduction of the pH - due to the lactic acidosis - and an
296 increase of the electrical conductivity, as shown in previous reports (Depreux, Grant, &
297 Gerrard, 2002; Fernandez, Neyraud, Astruc, & Sante, 2002; Krischek, Natter, Wigger,
298 & Wicke, 2011; Shen, Underwood, Means, McCormick, & Du, 2007).
299 Consequences of this calcium-related metabolic changes are often increasing drip loss
300 and higher meat lightness (L*), although the results found in the literature depend on the
301 particularities of every experiment (stress level and duration, animal's evaluation of the
302 situation), the intrinsic characteristics of the muscle (glycogen reserves, antioxidant
303 status) and the resulting *post mortem* rate of pH decline and protein denaturation. In our
304 work, the component L* did not show any significance for the analyzed factors, which
305 is in accordance with some reports (Channon et al., 2000; D'Souza, Dunshea, Warner,
306 & Leury, 1998; Hambrecht et al., 2005a) but contrary to others (Terlouw and Rybarczyk
307 2008; Boler et al., 2008; Edwards et al., 2010; Dokmanovic et al., 2015) which clearly
308 reflects the complexity of the processes involved. Nevertheless, other meat color traits,
309 such as a*, was significantly reduced in the Nn genotype ($P < 0.01$), which could be
310 result of higher *post mortem* protein denaturation and/or proteolysis (Kazemi, Ngadi
311 and Gariépy et al., 2011). This effect was significantly higher in males than in females,
312 and the same effect was observed for b* coordinate, for this reason there was a
313 significant interaction of sex and genotype on meat colour variables a* and b* (Table
314 1).

768
769
770
771 315 Another key quality trait, such as meat toughness, was significantly affected by RYR1
772
773 316 genotype ($P < 0.05$), with Nn animals exhibiting tougher meat (higher shear force)
774
775 317 along the process of meat aging (1 to 5 d *post mortem*, see Fig. 1), which agrees with
776
777 318 previous reports that described higher shear force and less tender meat in Nn than in
778
779 319 halothane free (NN) pigs (Channon et al., 2000; Fernández et al., 2002; Van den
780
781 320 Maagdenberg, Stinckens, Lefaucheur, Buys, & De Smet, 2008).
782
783 321 Furthermore, our results indicate increasing differences of shear force between
784
785 322 genotypes as the process of meat tenderization progressed (Fig. 1), although the
786
787 323 interaction between RYR1 genotype and aging time was not significant ($P = 0.737$).
788
789 324 Obviously, meat tenderness tended to increase as aging time increased in all meat types,
790
791 325 but the effect of the RYR1 mutation on meat toughness is probably related to
792
793 326 differences in the *post mortem* metabolism. All data recorded in this work (faster pH
794
795 327 decline, higher drip loss) indicate a fast *post mortem* metabolism in the muscle of Nn
796
797 328 pigs, confirmed in previous works, such as the one by Cheah, Cheah, & Krausgrill
798
799 329 (1995), who observed higher sarcoplasmic levels of calcium *in vivo* in the muscle from
800
801 330 Nn animals in contrast with NN, possibly causing a faster than normal rate of *post*
802
803 331 *mortem* muscle glycolysis in these pigs. In the same way, Depreux et al. (2002)
804
805 332 described a higher proportion of glycolytic fibres in the muscle of Nn genotype than
806
807 333 NN, expecting a more rapid *post mortem* pH decrease. This could imply a higher rate of
808
809 334 exhaustion of enzymes implicated in meat tenderization and therefore shorter
810
811 335 tenderization process. This agrees with previous histological and histochemical
812
813 336 investigations that have revealed increased fibre diameter and increased glycolytic
814
815 337 metabolic potential in the LT muscle of pigs with the RYR1 mutation, due to higher
816
817 338 proportion of the fast twitch glycolytic fibre type and lower of the slow twitch oxidative
818
819 339 type (Fiedler et al., 1999).
820
821
822

823
824
825
826

827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850
851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885

340

341 *3.2. Blood biochemical variables*

342 Sex affected several blood metabolites at slaughter (Table 2). Then, females showed
343 higher levels than males of glucose ($P < 0.05$), urea ($P < 0.001$), CRP ($P < 0.05$), Pig-
344 MAP ($P < 0.01$) and GPx ($P < 0.01$) and lower of lactate ($P < 0.05$).

345 Higher glucose level may indicate a higher stress response of females at slaughter, as it
346 is known that during psychological stress the organism feels threatened and gets ready
347 to respond to protect itself, then the glucose level in plasma increases due to the
348 secretion of hormones that leads to an increase on the hepatic glycogen breakdown and
349 gluconeogenesis (Becerril-Herrera et al., 2007; Mota-Rojas et al., 2009). There are a
350 number of studies that describe the increase of serum or plasma levels in glucose as a
351 consequence of stress in different animal species (see Becerril-Herrera et al., 2007) but
352 the effect of sex on the energetic profile is not clear as it may be affected by hormonal
353 differences. Our results are in accordance with the report by Mota-Roja et al. (2012)
354 who found increased concentration of glucose at exsanguinations in female pigs
355 subjected to acute stress, when compared to barrows and entire males.

356 Our data are also consistent with previous studies describing increased levels of APPs
357 such as CRP and Pig-MAP in plasma as consequence of stress in pigs (Murata 2007;
358 Piñeiro et al., 2007a, 2007b; Saco et al., 2003; Salamano et al., 2008).

359 When looking to differences of urea serum content within groups (Fig. 2a), it is
360 worthwhile to mention that increased urea concentration in females was consistent in
361 both NN and Nn groups, suggesting a faster catabolism of proteins, probably associated
362 to the above mentioned higher susceptibility of females to pre-slaughter stress.

363 The effect of the RYR1 genotype on variables such as creatinine ($P < 0.05$), CRP ($P <$
364 0.05), Pig-MAP ($P = 0.05$), CK ($P < 0.001$) and GPx ($P < 0.01$) was significant (Table

2). Heterozygous (Nn) pigs showed higher serum CK activity, which suggests increased muscle damage, and higher CRP concentration, that may indicate higher stress level and subsequent inflammation. To date, some APPs have been proposed as indicators of animal stress (Saco et al., 2003; Piñeiro et al., 2007a; Salamano et al., 2008; Marco-Ramell et al., 2011; Marco-Ramell et al., 2016), although the effect of stress on their serum concentration remains controversial, since it is difficult to distinguish it from the effect of trauma or subclinical infections.

On the other side, the higher GPx activity in homozygous (NN), but especially in females (there was significant S*G interaction, $P < 0.01$) suggests more potent antioxidant defenses in females, probably due to estrogen influence (Fig. 2b).

3.3. Muscle proteins

A total of 26 protein bands (201 to 20 kDa) were differentiated by SDS-PAGE gels in the muscle sarcoplasmic extracts, as shown in Figure 3, where band names are denoted by S of "sarcoplasmic" protein, followed by a number (1 to 26).

Table 3 gives the identification of protein bands with differential expression between treatments and Table 4 shows the effect of sex and genotype and its interaction on the abundance of these proteins.

3.3.1. Effect of sex

Myosin-binding protein C fast type, "MyBP-C" was overrepresented ($P < 0.05$) in the muscle of females. MyBP-C belongs to the myosin-binding protein C family, including fast- and slow-type isoforms, each of which is a myosin-associated protein found in the cross-bridge-bearing zone (C region) of sarcomeric A bands, where interaction between the thick and thin filaments occurs. Both structural and regulatory roles have been

proposed for MyBP-C, as it may modulate muscle contraction (Oakley, Hambly, Curmi, & Brown, 2004).

The presence of S6 (muscle-6-phosphofructokinase “PFK-M”) was also significantly affected by gender, with lower values in the muscle of females. PFK-M is the main rate-controlling enzyme of glycolysis, which catalyzes the transfer of a phosphoryl group from ATP to fructose-6-phosphate to yield ADP and fructose-1,6-bisphosphate. This enzyme is tightly regulated and responds to diverse molecules and signals by changing its catalytic activity and behaviour and is one of the few examples in which inhibition by the substrate occurs, as ATP may inhibit PFK at different levels, depending on the tissue metabolic state (Sola-Penna, Da Silva, Coelho, Marinho-Carvalho, & Zancan, 2010). Furthermore, lactate potentiate the inhibitory effects of ATP on PFK (Leite, Da Silva, Coelho, Zancan, & Sola-Penna, 2007). Then in our study underexpression of PFK-M in the muscle of females, that showed faster *post mortem* acidification, could be potentiated by inhibition due to lactate, although we can not discard a possible lower inherent PFK-M concentration in the muscle of females due to physiological differences between males and females.

3.3.2. Effect of genotype

Genotype affected the presence of four peptide bands, thus producing lower presence of S2 (MyBP-C, $P < 0.05$), S18 (glyceraldehyde-3-phosphate dehydrogenase “GAPDH”, $P < 0.01$) and S24 (containing two proteins: carbonic anhydrase “CAIII” and phosphoglycerate mutase-2 “PGM2”, $P < 0.05$) and higher of S23 (ENO3) in the muscle of Nn pigs (Table 4).

Changes of MyBP-C, which corresponds to the muscle fibre structure, could be due to the above mentioned differences of fibre type composition between RYR1 genotypes, while the other significant changes affected to metabolic enzymes (GAPDH, CAIII

and/or PGM2, ENO3) that showed significant correlation with the rate of *post mortem* muscle pH decline (pH-amplitude), being this relationship positive for GAPDH ($r=0.540$, $p < 0.01$) and CAIII/PGM2 ($r=0.410$, $P < 0.05$) and negative for ENO3 ($r=-0.541$, $P < 0.006$). This agrees with results from Gagaoua et al. (2015) who found negative relationship between ENO3 and pH decline in beef.

Lower GAPDH in the muscle of Nn pigs indicate lower glycolysis, which could be produced by an earlier depletion of muscle metabolites (glycogen) due to stress, as found by Fernandez et al. (2002). It is worthwhile to mention that GAPDH has recently been implicated in different non-metabolic processes, including transcription activation and initiation of apoptosis (Tarze et al., 2007). Moreover, GAPDH may act as a reversible metabolic switch under oxidative stress (Agarwal et al., 2012).

With respect to ENO3, it is a glycolytic enzyme that has been associated in beef with a faster *post mortem* muscle energy metabolism resulting in a faster pH decline (Gagaoua et al., 2015), and also has been correlated to beef colour stability (Gagaoua et al., 2015; Gagaoua, Terlouw, & Picard, 2017; Picard, Gagaoua, & Hollung, 2017) and to meat tenderization (Lametsch et al., 2003; Polati et al., 2012). Furthermore, ENO3 has been described as a hypoxic stress protein providing protection of cells by increasing anaerobic metabolism (Pancholi, 2001; Wulff, Jokumsen, Højrup, & Jessen, 2012). Then, it could be expected to find increased ENO3 in the muscle of pigs suffering higher stress at slaughter, that is, those from the Nn genotype.

The interpretation of changes found in the protein band S24 become difficult due to the co-migration of two proteins (CAIII and PGM2) and the resultant joint quantification, which is one of the difficulties of using 1D electrophoresis for protein separation. Band S24 showed significantly ($P<0.05$) lower abundance in the muscle extracts of Nn animals, and this difference was consistent regardless of sex (males and females), but

we could not dilucidate if both proteins (CAIII and PGM2) or only one of them had lower presence in Nn pigs. Anyway, lower CAIII (which functions as oxyradical scavenger and thus protects cells from oxidative damage) could be expected in the muscle of Nn pigs and would reflect lower level of the antioxidant defense, which agrees with the findings of Laville et al. (2009) who described reduced abundance of antioxidant proteins in the SM muscle of pigs with RYR1 mutation (nn genotype) compared with NN pigs, probably because the nn muscle was less oxidative and in consequence presented less antioxidative and repair capacities. The growing interest of meat scientist for the role of the balance between oxidative stress and antioxidant defense in the *post mortem* muscle is more than evident, and gives significant correlations with ultimate meat quality traits such as meat colour and tenderness (Laville et al., 2007, 2009; Jia et al., 2009; Ouali et al., 2013; Gagaoua et al., 2015, 2017; te Pas et al., 2017).

The other protein found in band S24 was PGM2, which catalyzes the interconversion of 2-phosphoglycerate and 3-phosphoglycerate in the glycolytic pathway and therefore it has a role of regulation of the energy balance and in the glycogen metabolism and glycolysis of the skeletal muscle (Fontanesi et al., 2008). This protein is encoded by a gene localized on porcine chromosome 18 (Fontanesi, Davoli, Nanni Costa, Scotti, & Russo, 2003) in a region where quantitative trait loci for drip loss, meat colour, fat deposition, lean content, muscle fiber diameter and carcass quality have been identified, and it has been described a significant association between PGM2 and drip loss in pigs (Fontanesi et al., 2003), so a lower PGM2 abundance in the muscle of Nn pigs, that showed higher drip loss, would be expected.

As a whole, the deficiency of GAPDH, PGM2 and/or CAIII and the increased amount of ENO3 in the muscle of Nn pigs after slaughter reflects an impairment of the

glycolysis function and a higher defense of the muscle cell to oxidative stress, which could be related to metabolic changes due to the RYR1 mutation, that causes a dysregulation of the calcium homeostasis and lead to neuromuscular disorders (Treves et al, 2005) and even can affect immunological and neuroendocrine response of pigs to stress (Ciepielewski et al., 2016). In our study we could not quantify changes in the abundance of muscle proteins involved in calcium homeostasis, such as sarcalumenin or calsequestrin-1, that were not separated in the 1D SDS-PAGE gels. These proteins have recently been detected by 2D-electrophoresis in mice muscle by Picard et al. (2016) who found that its abundance in the *Tibialis anterior* muscle (fast glycolytic) increased in the absence of Hsp27 (heat shock protein that has been described as beef tenderness biomarker, by the group of Picard).

3.3.3. Interaction of sex and genotype

The statistical analysis showed that there was a significant interaction between sex and genotype for two muscle proteins: S9 (albumin) and S26 (AK-1). That is, in this study, males tended to show higher muscle albumin expression than females, which could reflect physiological differences related to the function of albumin in the skeletal muscle, where it serves as a temporary amino acid storage site, maintains osmotic pressure and acts as a transporter for free fatty acids (Ellmerer et al., 2000), but the presence of the RYR1 mutation increased the albumin expression in the muscle of females and decreased it in males. Regarding AK-1, which catalyzes the reversible transfer of the terminal phosphate group between ATP and AMP and is a key enzyme in the muscle energetic homeostasis, it showed slightly higher level in Nn genotypes, which was more evident in males than in females, then showing significant interaction between sex and genotype. Our results show lower AK-1 level in Nn females, which

could be related with alterations of the muscle homeostasis as a result of higher stress response at slaughter, which is in agreement with a previous report from our group that showed that higher pre-slaughter stress produced lower presence of AK-1 in the *post mortem* muscle in pigs when mixed with unfamiliar animals (Oliván et al., 2016).

3.4. Multivariate analysis

Multivariate analysis was applied in order to obtain a synthetic assessment of the complex relationships between the variables best suited for factor analysis (KMO>0.6), that were: three meat quality traits (pH45, EC and drip), four serum metabolites (glucose, creatinine, CK and GPx) and three protein bands containing muscle proteins of the energy metabolism and antioxidant defense (GAPDH, ENO3 and CAIII/PGM2). The biplot obtained via PCA (Fig. 4) showed that PC1 and PC2 explained 62 % of the variability in the data.

The first principal component (PC1) distinguished in the positive side main variables related to poor meat quality: drip loss, EC and meat toughness (shear force at 5 days aging (WBSF-5d). Other variables with high loadings for PC1 were serum creatinine and CK, which indicate higher muscle damage at slaughter, and ENO3, a muscle protein that has been related to faster energy metabolism and faster pH decline in beef (Gagaoua et al., 2015) and also to hypoxic stress (Sedoris et al., 2010).

Furthermore, carcass temperature showed a positive correlation with PC1, which all together clearly indicates that the positive side of PC1 merged variables related to stress at slaughter. These characteristics corresponded to animals of the Nn genotype, mainly females, whose mean score showed high positive correlation to PC1 (Figure 4). By contrast, the negative side of the PC1 grouped meat variables indicating normal *post mortem* pH decline (higher pH at 45min), normal muscle glycolytic metabolism (higher

muscle GAPDH and PGM2 at slaughter) and higher muscle antioxidant defense (CAIII), that is, those variables that in general contribute to an appropriate process of muscle-to-meat conversion, being the NN genotype (males "M-NN" and females "F-NN") represented nearby.

The second PC aimed to distinguish in the positive side animals showing higher blood levels of glucose and GPx, that is, variables indicating stress, inflammation and antioxidant response at slaughter, which corresponded mainly to Females of the NN genotype.

Overall, these results show that the RYR1 mutation in heterozygosity contributed to reduce the ultimate meat quality (higher meat exudation and toughness) and that in some extent its effect was modulated by a higher stress response of Nn individuals at slaughter (higher serum level of creatinine and creatin kinase, compared to NN pigs). On the other hand, females showed faster muscle *post mortem* pH decline and produced more exudative meat than males, and also showed blood biochemical parameters at slaughter that seem to reflect a physiological response to stress (higher glucose and GPx).

Furthermore, from a proteomic perspective, these results allowed the identification of key proteins involved in the *post mortem* glycolytic pathway (GAPDH, PGM2, ENO3) and the antioxidant defense (CAIII) of the muscle that contribute to the process of meat quality acquisition and are influenced by pre-slaughter stress. These proteins have a relevant role in the *post mortem* muscle metabolism and most of them have already been identified as biomarkers of meat quality and animal stress (Laville et al., 2007, 2009; Guillemin, Bonnet, Jurie, & Picard, 2011; Gagaoua et al., 2015, 2017; Oliván et al., 2016).

These results contribute to progress towards the comprehensive identification of proteins linked to the process of meat quality acquisition, being ultimately modulated by the animal's stress reaction at slaughter. Knowing the biological mechanism underlying this process opens up the possibility of monitoring and predicting the resulting changes. Once this is known, these potential protein biomarkers must follow a process of evaluation and validation (Naylor, 2003; Te Pas, Hoekman & Smits, 2011; Picard & Gagaoua, 2017), so further research is needed on a larger data set.

4. Conclusions

Pork quality development is largely governed by the rate and extent of *post mortem* muscle metabolism, which is affected by animal factors like sex and RYR1 genotype, with influence as well in the modulation of the animal's individual susceptibility to pre-slaughter stress.

The results of this study showed that the sex and the RYR1 genotype affected several blood biochemical parameters at slaughter and some muscle enzymes with key role on the subsequent process of muscle-to-meat conversion, showing Nn females more susceptibility to stress, with detrimental effect on meat quality.

These differences may be monitored by protein biomarkers related to the fibre composition, the *post mortem* glycolytic pathway and the antioxidant defense of the muscle. However, it is worthwhile to mention that the complex nature of the processes that underlie the *post mortem* meat quality development and the high diversity of factors that may influence the animal's susceptibility to stress at slaughter makes difficult to find universal biomarkers. Then, more research is needed in order to apply combined "omics" techniques that allow the identification of key protein biomarkers and to validate them in different breeds and management systems.

Acknowledgements

This study was funded by projects AGL 2011-30598-C03 (Ministerio de Economía y Competitividad, Spain), FISS-13-RD12/0043/0030 and FISS-14-PI13/02741 (Instituto de Salud Carlos III, Spain). M. Oliván, Y. Potes and A. Coto-Montes are members of the Research Team "cellular Response to Oxidative Stress (cROS)" of University of Oviedo. Y. Potes thanks the FISS pre-doctoral fellowship from the Ministerio de Economía y Competitividad (Instituto de Salud Carlos III). We are grateful to V. Fernández-Suárez for collaboration in analytical procedures.

References

Agarwal, A. R., Zhao, L., Sancheti, H., Sundar, I. K., Rahman, I., & Cadenas, E. (2012). Short-term cigarette smoke exposure induces reversible changes in energy metabolism and cellular redox status independent of inflammatory responses in mouse lungs. *American Journal of Physiology: Lung Cell Molecular Physiology, 303*, 889-898.

Ampuero Kragten, S., & Gil, M. (2015). Chapter 5: Instrumental tenderness - shear force. In M. Font-i-Furnols, M. Čandek-Potokar, C. Maltin, M. Prevolnik Povše (Eds.), *A handbook of reference methods for meat quality assessment* (pp.45-54). Farm Animal Imaging Cost Action FA1102.

Becerril-Herrera, M., Alonso-Spilsbury, M., Lemus-Flores, C., Guerrero-Legarreta, I., Olmos-Hernández, A., Ramírez-Necoechea, R., & Mota-Rojas, D. (2007). CO2 stunning may compromise swine welfare compared with electrical stunning. *Meat Science, 81,* 233-237.

Blanchard, P. J., Warkup, C. C., Ellis, M., Willis, M. B., & Avery, P. (1999). The influence of the proportion of Duroc genes on growth, carcass and pork eating quality characteristics. *Animal Science, 68*, 495-501.

Boler, D. D., Dilger, A. C., Bidner, B. S., Carr, S. N., Eggert, J. M., Day, J. W., Ellis, M., McKeith, F. K., & Killefer, J. (2010). Ultimate pH explains variation in pork quality traits. *Journal of Muscle Foods, 21,* 119-130.

Channon, H. A., Kerr, M. G., & Walker, P. J. (2004). Effect of Duroc content, sex and ageing period on meat and eating quality attributes of pork loin. *Meat Science, 66*, 881-888.

Channon, H. A., Payne, A. M., & Warner, R. D. (2000). Halothane genotype, pre-slaughter handling and stunning method all influence pork quality. *Meat Science, 56,* 291-299.

Cheah, K. S., Cheah, A. M., & Krausgrill, D. I. (1995). Variations in meat quality in live halothane heterozygotes identified by biopsy samples of M. longissimus dorsi. *Meat Science, 39*, 293-300.

Ciepielewski, Z. M., Stojek, W., Borman, A. Myślińska, D., Pałczyńska, P., & Kamyczek, M. (2016). The effects of ryanodine receptor (RYR1) mutation on natural killer cell cytotoxicity, plasma cytokines and stress hormones during acute intermittent exercise in pigs. *Research in Veterinary Science, 105*, 77-86.

Cisneros, F., Ellis, M., Mckeith, F. K., McCaw, J., & Fernando, R. L. (1996). Influence of slaughter weight on growth and carcass characteristics, commercial cutting and curing yields, and meat quality of barrows and gilts from two genotypes. *Journal of Animal Science, 74*, 925-933.

Dalmau, A., Temple D., Rodriguez, P., Llonch, P., & Velarde, A. (2009). Application of the Welfare Quality® protocol at pig slaughterhouses. *Animal Welfare*, *18,* 497-505.

D'Eath R. B., Turner, S. P., Kurt, E., Evans, G., Thölking, L., Looft, H., Wimmers, K., Murani, E., Klont, R., Foury, A., Ison, S. H., Lawrence, A. B., & Mormède, P. (2010). Pig's agressive temperament affects pre-slaughter mixing aggression, stress and meat quality. *Animal, 4,* 604-616.

Depreux, F. F. S., Grant, A. L., & Gerrard, D. E. (2002). Influence of halothane genotype and body-weight on myosin heavy chain composition in pig muscle as related to meat quality. *Livestock Production Science, 73*, 265-273.

Dokmanovic, M., Baltic, M. Z., Duric, J., Ivanovic, J., Popovic, L., Todorovic, M., Markovic, R., & Pantic, S. (2015). Correlations among Stress Parameters, Meat and Carcass Quality Parameters in Pigs. *Asian-Australasian Journal of Animal Sciences, 28,* 435-441.

D'Souza, D. N., Dunshea, R. R., Warner, R. D., & Leury, B. J. (1998). The effect of handling pre-slaughter and carcass processing rate post-slaughter on pork quality. *Meat Science, 50*, 429-437.

D'Souza, D. N., & Mullan, B. P. (2002). The effect of genotype, sex and management strategy on the eating quality of pork. *Meat Science, 60*, 95-101.

Edwards, L. N., Engle, T. E., Correa, J. A., Paradis, M. A., Grandin, T., & Anderson, D. B. (2010a). The relationship between exsanguination blood lactate concentration and carcass quality in slaughter pigs. *Meat Science, 85,* 435-440.

Edwards, L. N., Grandin, T., Engle, T. E., Porter, S. P., Ritter, M. J., Sosnicki, A. A., & Anderson, D. B. (2010b). Use of exsanguination blood lactate to assess the quality of pre-slaughter pig handling. *Meat Science, 86*, 384-390.

Ellmerer, M., Schaupp, L., Brunner, G. A., Sendlhofer, G., Wutte, A., Wach, P., & Pieber, T. R. (2000). Measurements of interstitial albumin in human skeletal muscle and adipose tissue by open-low microperfusion. *American Journal of Physiology. Endocrinology and Metabolism*, *285*, E352-E356.

E.U. (2010). Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. *Official Journal of the European Communities, L276*, 33-79.

Fàbrega, E., Manteca, X., Font, J., Gispert, M., Carrión, D., Velarde, A., Ruiz-de-la-Torre, J. L., & Diestre, A. (2004). A comparison of halothane homozygous negative and

positive pietrain sire lines in relation to carcass and meat quality, and welfare traits. *Meat Science, 66,* 777-787.

Fàbrega, E., Velarde, A. Cros, J., Gispert, M., Suarez, P., Tibau, J., & Soer, J. 2010. Effect of vaccination against gonodotroping-releasing hormone, using Improvac®, on growth performance, body composition, behaviour and acute phase proteins. *Livestock Science, 132*, 53-59.

Fernandez, X., Neyraud, E., Astruc, T., & Sante, V. (2002). Effects of halothane genotype and pre-slaughter treatment on pig meat quality. Part 1. Post mortem metabolism, meat quality indicators and sensory traits of m. Longissimus lumborum. *Meat Science, 62,* 429-437.

Fiedler, I., Ender, K., Wicke, M., Maak, S., Lengerken, G. V., & Meyer, W. (1999). Structural and functional characteristics of muscle fibres in pigs with different malignant hyperthermia susceptibility (MHS) and different meat quality. *Meat Science, 53*, 9-15.

Franco, D., Mato, A., Salgado, F. J., López-Pedrouso, M., Carrera, M., Bravo, S., Parrado, M., Gallardo, J. M., & Zapata, C. (2015). Tackling proteome changes in the longissimus thoracis bovine muscle in response to pre-slaughter stress. *Journal of Proteomics, 122*, 73-85.

Fujii, J., Otsu, K., Zorzato, F., De Leon, S., Khanna, V. K., Weiler, J. E., O'Brien, P. J., & MacLennan, D. H. (1991). Identification of a mutation in porcine ryanodine receptor associated with malignant hyperthermia. *Science, 253,* 448-451.

Gagaoua, M., Terlouw, E. M. C., Micol, D., Boudjellal, A., Hocquette, J. F., & Picard, B. (2015). Understanding early post-mortem biochemical processes underlying meat color and pH decline in the Longissimus thoracis muscle of young blond d'Aquitaine bulls using protein biomarkers. *Journal of Agricultural and Food Chemistry, 63*, 6799-6809.

Gagaoua, M., Terlouw, E. M. C., Picard, B. (2017). The study of protein biomarkers to understand the biochemical processes underlying beef color development in young bulls. *Meat Science, 134*, 18-27.

Gispert, M., Àngels Oliver, M., Velarde, A., Suarez, P., Pérez, J., & Font i Furnols, M. (2010). Carcass and meat quality characteristics of immunocastrated male, surgically castrated male, entire male and female pigs. *Meat Science, 85*, 664-670.

Guillemin, N., Bonnet, M., Jurie, C., & Picard, B. (2011). Functional analysis of beef tenderness. *Journal of Proteomics, 75*, 352-365.

Hambrecht, E., Eissen, J. J., Newman, D. J., Smits, C. H. M.,. den Hartog, L. A., & Verstegen, M. W. A. (2005a). Negative effects of stress immediately before slaughter on pork quality are aggravated by suboptimal transport and lairage conditions. *Journal of Animal Science, 83*, 440-448.

Hambrecht, E., Eissen, J. J., Newman, D. J., Smits, C. H. M., Verstegen, M. W. A., & den Hartog, L. A. (2015b). Preslaughter handling effects on pork quality and glycolytic potential in two muscles differing in fiber type composition. *Journal of Animal Science, 83*, 900-907.

Havlis, J., Thomas, H., Sebela, M., & Shevchenko, A. (2003). Fast-response proteomics by accelerated in-gel digestion of proteins. *Analytical Chemistry, 75*, 1300-1306.

Hollung, K., Veiseth, E., Jia, X., Færgestad, E. M., & Hildrum, K. I. (2007). Application of proteomics to understand the molecular mechanisms behind meat quality. *Meat Science, 77*, 97-104.

Jami, M. S., Barreiro, C., García-Estrada, C., & Martín, J. F. (2010). Proteome analysis of the penicillin producer Penicillium chrysogenum: characterization of protein changes during the industrial strain improvement. *Molecular & Cellular Proteomics, 9*, 1182-1198.

Jia, X., Veiseth-Kent, E., Grove, H., Kuziora, P., Aass, L., Hildrum, K. I., & Hollung, K. (2009). Peroxiredoxin-6_A potential protein marker for meat tenderness in bovine longissimus thoracis muscle. *Journal of Animal Science, 87,* 2391-2399.

Kazemi, S., Ngadi, M. O., & Gariépy, C. (2011). Protein Denaturation in Pork Longissimus Muscle of Different Quality Groups. *Food and Bioprocess Technology, 4*, 102-106.

Krischek, C., Natter, R., Wigger, R., & Wicke, M. (2011). Adenine nucleotide concentrations and glycolytic enzyme activities in longissimus muscle samples of different pig genotypes collected before and after slaughter. *Meat Science, 89*, 217-220.

Lametsch, R., Karlsson, A., Rosenvold, K., Andersen, H. J., Roepstorff, P., & Bendixen, E. (2003). Postmortem proteome changes of porcine muscle related to tenderness. *Journal of Agricultural and Food Chemistry, 51*, 6992-6997.

Laville, E., Sayd, T., Terlouw, C., Chambon, Ch., Damon, M., Larzul, C., Leroy, P., Glénisson,J., & Chérel, P. (2007). Comparison of Sarcoplasmic Proteomes between Two Groups of Pig Muscles Selected for Shear Force of Cooked Meat. *Journal of Agricultural and Food Chemistry, 55*, 5834-5841.

Laville, E., Sayd, T., Terlouw, C., Blinet, S., Pinguet, J., Fillaut, M., Glénisson, J., & Chérel, P. (2009). Differences in Pig Muscle Proteome According to HAL Genotype: Implications for Meat Quality Defects. *Journal of Agricultural and. Food Chemistry, 57*, 4913-4923.

Leite, T. C., Da Silva, D., Coelho, R. G., Zancan, P.,& Sola-Penna, M. (2007). Lactate favours the dissociation of skeletal muscle 6-phosphofructo-1-kinase tetramers down-regulating the enzyme and muscle glycolysis. *Biochemical Journal, 408*, 123-130.

Marco-Ramell, A., Pato, R., Peña, R., Saco, Y., Manteca, X., Ruiz de la Torre, J. L., Bassols, A. (2011). Identification of serum stress biomarkers in pigs housed at different stocking densities. *Veterinary Journal, 190*, 66-71.

Marco-Ramell, A., Arroyo, L., Peña, R., Pato, R., Saco, Y., Fraile, L., Bendixen, E., & Bassols, A. (2016). Biochemical and proteomic analyses of the physiological response induced by individual housing in gilts provide new potential stress markers. *BMC Veterinary Research, 12*, 265.

Mota-Rojas, D., Herrera, M. B., Trujillo-Ortega, M. E., Alonso-Spilsbury, M., Flores-Peinado, S. C., & Guerrero-Legarreta, I. (2009). Effects of pre-slaughter transport, lairage and sex on pig chemical serologic profiles. *Journal of Animal Veterinary Advances, 8,* 246-250.

Mota-Rojas, D., Becerril-Herrera, M., Roldan-Santiago, P., Alonso-Spilsbury, M., Flores-Peinado, S., Ramírez-Necoechea, R., Ramírez-Telles, J. A., Mora-Medina, P., Pérez, M., Molina, E. Soní, E., & Trujillo-Ortega, M. E. (2012). Effects of long distance transportation and CO_2 stunning on critical blood values in pigs. *Meat Science, 90*, 893-898.

Murata, H. (2007). Stress and acute phase protein response: An inconspicuous but essential linkage. *Veterinary Journal, 173*, 473-474

Naylor, S. (2003). Biomarkers: current perspectives and future prospects. *Expert Review of Molecular Diagnostics, 3*, 525-529.

Oakley, C., Hambly, B. D., Curmi, P. M. G., & Brown, L. J. (2004). Myosin binding protein C: Structural abnormalities in familial hypertrophic cardiomyopathy. *Cell Research, 14*, 95-110.

Oliván, M., Fernández-Suárez, V., Díaz-Martínez, F., Sierra, V., Coto-Montes, A., de Luxán-Delgado, B., Peña, R., Bassols, A., Fàbrega, E., Dalmau, A., & Velarde, A. (2016). Identification of Biomarkers of Stress in Meat of Pigs Managed under Different Mixing Treatments. *British Biotechnology Journal*, *11*, 1-13.

Ouali, A., Gagaoua, M., Boudida, Y., Becila, S., Boudjellal, A., Herrera-Mendez, C., & Sentandreu, M. A. (2013). Biomarkers of meat tenderness: Present knowledge and perspectives in regards to our current understanding of the mechanisms involved. *Meat Science, 95,* 854-870.

Pancholi, V. (2001). Multifunctional alpha-enolase: its role in diseases*. Cellular and Molecular Life Sciences, 58*, 902-920.

Picard, B., & Gagaoua, M. (2017). Chapter 11: Proteomic Investigations of Beef Tenderness. In: M. Colgrave (Ed.), *Proteomics in Food Science: from farm to fork* (pp. 177-197). Academic Press.

Picard, B., Gagaoua, M., & Hollung, K. (2017). Chapter 12: Gene and Protein Expression as a Tool to Explain/Predict Meat (and Fish) Quality. In P. Purslow (Ed.), *New Aspects of Meat Quality: from Genes to Ethics* (pp.321-354). Netherlands: Elsevier Science.

Picard, B., Kammoun, M., Gagaoua, M., Barboiron, Ch., Meunier, B., Cambon, Ch., & Cassar-Malek, I. (2016). Calcium Homeostasis and Muscle Energy Metabolism Are Modified in HspB1-Null Mice. *Proteomes, 4,* 17.

Piñeiro, M., Piñeiro, C., Carpintero, R., Morales, J., Campbell, F. M., Eckersall, P. D., Toussaint, M. J. M., & Lampreave, F. (2007a). Characterisation of the pig acute phase protein response to road transport. *The Veterinary Journal, 173*, 669-674.

Piñeiro, C., Piñeiro, M., Morales, J., Carpintero, C., Campbell, F. M., Eckersall, P. D., Toussaint, M. J. M., Alava, M. A., & Lampreave, F. (2007b). Pig acute-phase protein levels after stress induced by changes in the pattern of food administration. *Animal, 1,* 133-139.

Polati, R., Menini, M., Robotti, E., Millioni, R., Marengo, E., Novelli, E., Balzan, S., Cecconi, D. (2012). Proteomic changes involved in tenderization of bovine Longissimus dorsi muscle during prolonged ageing. *Food Chemistry,135*, 2052-69.

Rasmussen, A., & Andersson, M. (1996). New method for determination of drip loss in pork muscles. *Paper presented at the Proceedings of the 42th International Congress of Meat Science and Technology*, pp. 286-287. Lillehammer, Norway 1-6/09/1996.

Roberts, S. A., Schaefer, A. L., Murray, A. C., & Thibault, L. (1998). Fall and winter hormone concentrations related to stress in pigs identified as normal and carrier for stress susceptibility. *Chronobioly International Journal, 15*,275-281.

Salamano, G., Mellia, E., Candiani, E., Ingravalle, F., Bruno, R., Ru, G. & Doglione, L. (2008). Changes in haptoglobin, C-reactive protein and pig-MAP during a housing period following long distance transport in swine. *The Veterinary Journal, 177*, 110-115.

Saco, Y., Docampo, M. J., Fàbrega, E., Manteca, X., Diestre, A., Lampreave, F., & Bassols, A. (2003). Effect of transport stress on serum haptoglobin and Pig-MAP in pigs. *Animal Welfare, 12*, 403-409.

Sedoris, K. C., Thomas, S. D., & Miller, D. M. (2010). Hypoxia induces differential translation of enolase/MBP-1. *BMC Cancer, 10,* 157.

Shen, Q. W., Underwood, K. R., Means, W. J., McCormick, R. J., & Du, M. (2007). The halothane gene, energy metabolism, adenosine monophosphate-activated protein kinase, and glycolysis in postmortem pig longissimus dorsi muscle. *Journal of Animal Science, 85*,1054-1061.

Sierra, V., Fernández-Suárez, V., Castro, P., Osoro, K., Vega-Naredo, I., García-Macia, M., Rodríguez-Colunga, M. J., Coto-Montes, A. & Oliván, M. (2012). Identification of biomarkers of meat tenderisation and its use for early classification of Asturian beef into

fast and late tenderizing meat. *Journal of the Science of Food and Agriculture, 92*, 2727-2740.

Sola-Penna, M., Da Silva, D., Coelho, W. S., Marinho-Carvalho, M. M., & Zancan, P. (2010). Regulation of Mammalian Muscle Type 6-Phosphofructo-1-kinase and Its Implication for the Control of the Metabolism. *IUBMB Life, 62*, 791-796.

Tarrant, P. V. (1990). Transportation of cattle by road. *Applied Animal Behaviour Science, 28,* 153-170.

Tarze, A., Deniaud, A., Le Bras, M., Maillier, E., Molle, D., Larochette, N., Zamzami, N., Jan, G., Kroemer, G., & Brenner, C. (2007). GAPDH, a novel regulator of the pro-apoptotic mitochondrial membrane permeabilization. *Oncogene, 26,* 2606-2620.

Terlouw, C. (2005). Stress reactions at slaughter and meat quality in pigs: Genetic background and prior experience: A brief review of recent findings. *Livestock Production Science, 94*, 125-135.

Terlouw, C., & Rybarczyk, P. (2008). Explaining and predicting differences in meat quality through stress reactions at slaughter: The case of Large White and Duroc pigs. *Meat Science, 79,* 795-805.

tePas M. F. W., Hoekman, A. J. W., & Smits, M. A. (2011). Biomarkers as management tools for industries in the pork production chain. *Journal on Chain and Network Science, 11,* 155-166.

tePas, M. F. W., Lebret, B., & Oksbjerg, N. (2017). Measurable biomarkers linked to meat quality from different pig production systems. *Archives Animal Breeding, 60*, 271-283.

Treves, S., Anderson, A. A., Ducreux, S., Divet, A., Bleunven, Ch., Grasso, C., Paesant, S., & Zorzato, F. (2005). Ryanodine receptor 1 mutations, dysregulation of calcium homeostasis and neuromuscular disorders. *Neuromuscular Disorders, 15*, 577-587.

Van den Maagdenberg, K., Stinckens, A., Lefaucheur, L., Buys, N., & De Smet, S. (2008). The effect of mutations in the insulin-like growth factor-II and ryanodine receptor-1 genes on biochemical and histochemical muscle fibre characteristics in pigs. *Meat Science, 79*, 757-766.

Wulff, T., Jokumsen, A., Højrup, P., & Jessen, F. (2012). Time-dependent changes in protein expression in rainbow trout muscle following hypoxia. *Journal of Proteomics, 75,* 2342-2351.

Table 1 Least squares means and the effect of sex (S) and RYR1 genotype (G) and its interaction (S*G) on carcass and meat quality traits.

Sex	Male		Female			*P*-value		
Genotype	NN	Nn	NN	Nn	SEM[1]	S	G	S*G
Temperature (ºC)	37.37	37.38	37.10	39.47	1.386	0.7173	0.1332	0.2221
Skin lesions	0.33	0.67	0.5	0.33	0.552	0.6034	0.6034	1.000
pH45	6.57	6.32	6.42	6.13	0.171	0.0021	<.0001	0.3318
pH24	5.50	5.50	5.44	5.43	0.070	0.2909	0.2558	0.6532
pH amplitude (pH45-pH24)	1.075	0.820	0.987	0.703	0.094	0.2881	0.0102	0.8824
EC[2] (mS)	4.11	6.12	5.94	7.20	2.099	0.1088	0.0144	0.3219
Drip loss (%)	4.48	5.48	4.92	7.21	1.679	0.1887	<.0001	0.9829
L*	50.54	49.16	49.47	50.92	2.044	0.5354	0.553	0.0758
a*	6.97[c]	6.33[a]	6.77[b]	6.56[b]	0.692	0.8748	0.0064	0.0403
b*	2.18[b]	1.54[a]	2.05[b]	2.24[b]	0.734	0.7904	0.109	0.015
WBSF[3]-1d	4.91	5.10	4.17	5.19	0.920	0.3216	0.0326	0.9375
WBSF[3]-3d	4.22	4.22	3.72	4.54	0.706	0.655	0.0029	0.8014
WBSF[3]-5d	3.80	4.01	3.60	4.70	0.598	0.5445	0.0005	0.5598

Means in the same row followed by different superscripts are significantly different at $P < 0.05$.
[1]SEM: standard error of means
[2]EC: electrical conductivity
[3]WBSF: Warner-Braztler shear force at 1, 3 or 5 days *post mortem*

Table 2. Least squares means and the effect of sex (S) and RYR1 genotype (G) and its interaction (S*G) on serum biochemical variables at slaughter.

Sex	Male		Female			*P*-value		
Genotype	NN	Nn	NN	Nn	SEM[1]	S	G	S*G
Glucose, mg/dL	286.25	330.51	366.91	349.62	14.676	0.019	0.523	0.138
Lactate, mmol/L	10.61	10.37	8.53	9.35	0.432	0.013	0.622	0.377
Creatinine, mg/dL	1.88	2.03	2.00	2.06	0.034	0.123	0.035	0.329
Urea, mg/dL	27.08	25.31	37.90	32.69	1.473	<0.001	0.097	0.409
Total proteins, g/dL	6.84	6.66	6.85	6.82	0.066	0.396	0.258	0.434
Triglycerids, mg/dL	47.92	51.75	49.64	55.25	2.283	0.401	0.155	0.806
Cholesterol, mg/dL	90.33	87.43	92.15	95.64	1.811	0.053	0.919	0.219
HDL-chol[2], mmol/L	1.14	1.14	1.16	1.21	0.017	0.072	0.238	0.238
LDL-chol[3], mmol/L	1.35	1.26	1.32	1.33	0.031	0.602	0.304	0.288
NEFAs[4], mmol/L	0.080	0.082	0.085	0.067	0.008	0.657	0.426	0.342
BHB[5], mmol/L	0.072	0.085	0.081	0.077	0.005	0.933	0.511	0.279
Haptoglobin, mg/mL	0.174	0.408	0.523	0.474	0.085	0.091	0.437	0.245
CRP[6], µg/mL	4.35	10.05	10.56	12.35	1.246	0.018	0.039	0.261
Pig-MAP, mg/mL	0.62	0.58	0.96	0.72	0.049	0.001	0.050	0.163
CK[7], U/L	1844.58	3024.42	1920.36	4368.08	277.388	0.065	<0.001	0.125
GPx[8], U/L	8321.42[a]	8855.58[a]	14481.55[b]	8670.33[a]	683.073	0.003	0.009	0.002
Cortisol, ng/mL	39.03	38.95	30.92	43.37	2.869	0.670	0.139	0.134

Means in the same row followed by different superscripts are significantly different at $P < 0.05$.
[1]SEM: standard error of means
[2] HDL-chol: High density lipoproteins-cholesterol
[3] LDL-chol: High density lipoproteins-cholesterol
[4] NEFAs: non-esterified fatty acids
[5] BHB: β-hydroxybutyrate
[6] CRP: C-reactive protein
[7] CK: creatine kinase
[8] GPx: glutathione peroxidase

Table 3: Protein identification in noticeable bands of sarcoplasmic extracts separated by SDS-PAGE acrilamide gels

Band [MWe[1]]	Identification	Accession no.[2]	MOWSE[3] scores	Sequence Coverage (%)	Matched Queries	MWt[4]
S2 (175.6 kDa)	Myosin-binding protein C, fast-type [Sus scrofa]: **FastMyBP-C**	gi|335290041	111	14	13	128.4
S6 (86.8 kDa)	Muscle 6-phosphofructokinase [Sus scrofa]: **PFK-M**	gi|95117652	524	35	22	82.4
S9 (61.7 kDa)	**Albumin** [Sus scrofa]	gi|833798	808	38	20	71.4
S18 (32.5 kDa)	Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating): (EC 1.2.1.12)- pig: **GAPDH**	gi|65987	767	55	14	35.9
S23 (26.3 kDa)	β-enolase [Bos taurus]: **ENO3**	gi|77736349	282	32	9	47.4
S24 (25.3 kDa)	Carbonic anhydrase 3 [Sus scrofa]: **CAIII**	gi|56711366	858	76	21	29.7
	Phosphoglycerate mutase-2 [Sus scrofa]: **PGM2**	gi|201066358	400	56	13	28.8
S26 (20.5 kDa)	Adenylate kinase isoenzyme 1 [Sus scrofa]: **AK1**	gi|350579686	539	65	15	21.7

[1]MWe is the experimental molecular weight (kDa)
[2]Accession number correspond to NCBInr database
[3]The MOWSE score is a numeric descriptor of the likelihood that the identification is correct. Protein scores greater than 69 are significant ($P < 0.05$)
[4] MWt is the theoretical molecular weight (kDa)

Table 4. Least squares means and the effect of sex (S), RYR1 genotype (G) and its interaction (S*G) on the expression of noticeable proteins of the muscle sarcoplasmic extracts (optical density, in arbitrary units).

Sex	Male		Female			*P*-value		
Genotype	NN	Nn	NN	Nn	SEM[1]	S	G	S*G
Fast MyBP-C	0.217	0.168	0.370	0.223	0.034	0.048	0.044	0.272
PFK-M	1.328	1.498	0.854	0.790	0.144	0.006	0.911	0.466
Albumin	2.688[b]	2.329[b]	1.881[a]	2.441[b]	0.119	0.081	0.385	0.006
GAPDH	14.065	13.109	14.398	12.235	0.367	0.710	0.009	0.313
ENO3	0.379	1.049	0.667	0.934	0.124	0.704	0.017	0.211
CAIII/PGM2	7.967	7.140	7.907	7.111	0.204	0.963	0.012	0.870
AK-1	2.085[a]	1.985[a]	1.979[a]	2.160[b]	0.046	0.658	0.591	0.046

Means in the same row followed by different superscripts are significantly different at $P < 0.05$.
[1]SEM: standard error of means

Figure captions:

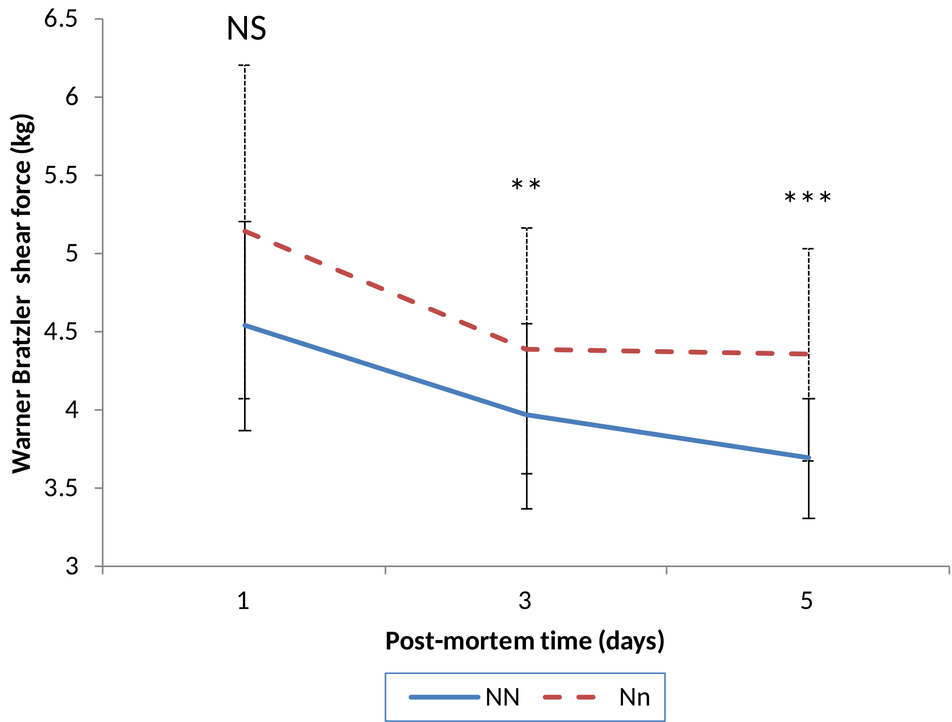
Figure 1. Effect of RYR1 mutation (NN: halothane free, Nn: gene mutation carrier) on the *post mortem* evolution of meat toughness, measured as maximum Warner Bratzler shear force (means ± S.E.). Significances: **: $P < 0.01$, ***: $P < 0.001$, NS: $P > 0.05$.

Figure 2. Urea (a) and GPx (b) levels in serum (means ± S.E.) in the four studied treatments (M: male, F: female, NN: halothane free, Nn: gene mutation carrier).

Figure 3. SDS-PAGE gel image of sarcoplasmic extracts of the LD muscle in the four treatments (M-NN, M-Nn, F-NN, F-Nn). Band names are denoted by S (sarcoplasmic protein) followed by a number.

Figure 4. PCA biplot of meat quality traits and stress biomarkers. Mean scores for animal treatments (M-NN, M-Nn, F-NN, F-Nn) are shown in squares.

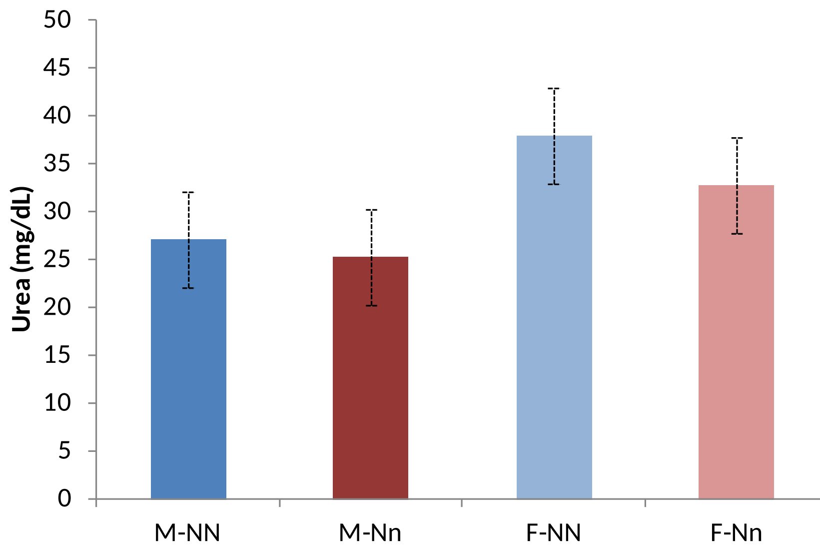
2479
2480
2481 955 Figure 1.
2482
2483 956
2484
2485 957
2486
2487



2488 2489 2490 2491 2492 2493 2494 2495 2496 2497 2498 2499 2500 2501 2502 2503 2504 2505 2506 2507 2508
2509 958
2510
2511 959
2512
2513 960
2514 2515 2516 2517 2518 2519 2520 2521 2522 2523 2524 2525 2526 2527 2528 2529 2530 2531 2532 2533

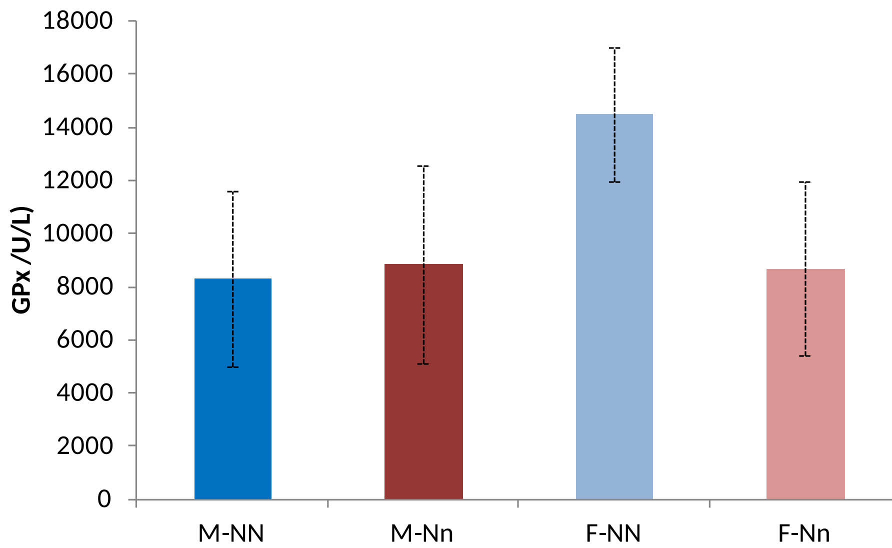
2534 2535 2536 2537

2538
2539
2540 961 Figure 2.
2541
2542 962 a)
2543



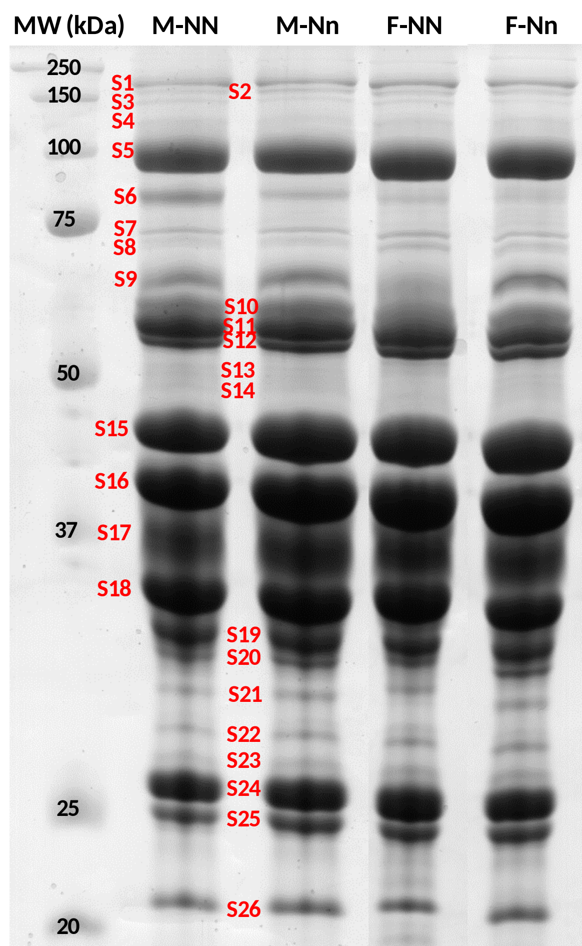
963

964 b)



965

966

2597
2598
2599 967 Figure 3.
2600
2601 968
2602
2603
2604
2605


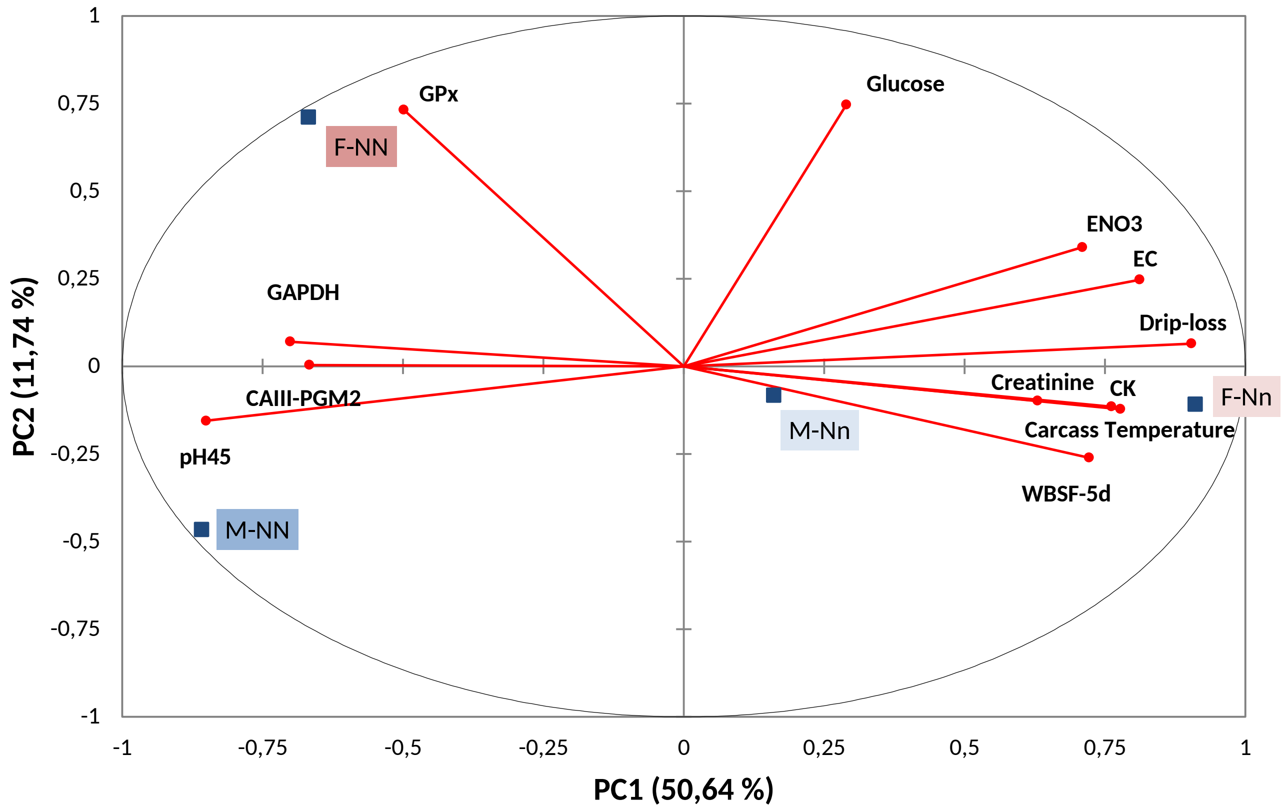
2630 969
2631
2632 970
2633
2634
2635
2636
2637
2638
2639
2640
2641
2642
2643
2644
2645
2646
2647
2648
2649
2650
2651
2652
2653
2654
2655

2656
2657
2658
2659
2660
2661
2662
2663
2664
2665
2666
2667
2668
2669
2670
2671
2672
2673
2674
2675
2676
2677
2678
2679
2680
2681
2682
2683
2684
2685
2686
2687
2688
2689
2690
2691
2692
2693
2694
2695
2696
2697
2698
2699
2700
2701
2702
2703
2704
2705
2706
2707
2708
2709
2710
2711
2712
2713
2714

971 Figure 4.

972

973



974
975 Variables: EC: Electrical conductivity; WBSF-5d: Warner Bratzler shear force at 5 days post mortem; CK: Creatine
976 kinase; GPx: Glutathione peroxidase; CAIII: Carbonic anhydrase; PGM2: Phosphoglycerate mutase-2; GAPDH:
977 Glyceraldehyde-3-phosphate dehydrogenase; ENO3: β-enolase
978 Treatments: M-NN (male-halothane free), M-Nn (male-gene mutation carrier), F-NN (female-halothane free) and F-
979 Nn (female-gene mutation carrier).
980

TABLES FOR ON-LINE SUPPLEMENTARY MATERIAL

Table: Protein expressions for the whole set of protein bands separated by SDS-PAGE acrilamide gels (optical density, in arbitrary units).

Sex	Male		Female		Significance		
Genotype	NN	Nn	NN	Nn	S	G	S*G
B1 (210.6 kDa)	0.539	0.717	0.485	0.613	NS	NS	NS
B2 (175.6 kDa)	0.217	0.169	0.370	0.223	*	*	NS
B3 (145.1 kDa)	0.226	0.225	0.126	0.158	NS	NS	NS
B4 (114.1 kDa)	0.209	0.322	0.257	0.325	NS	NS	NS
B5 (97.8 kDa)	4.925	6.376	5.756	5.847	NS	NS	NS
B6 (86.8 kDa)	1.328	1.498	0.854	0.789	**	NS	NS
B7 (73.8 kDa)	0.460	0.443	0.383	0.546	NS	NS	NS
B8 (70.5 kDa)	0.309	0.369	0.370	0.372	NS	NS	NS
B9 (61.7 kDa)	2.688	2.330	1.881	2.441	NS	NS	**
B10-11-12[1] (56.8-53.5 kDa)	11.038	10.999	11.782	11.287	NS	NS	NS
B13 (49.9 kDa)	0.617	0.403	0.476	0.501	NS	NS	NS
B14 (48.2 kDa)	0.082	0.326	0.092	0.934	NS	NS	NS
B15 (43.5 kDa)	12.837	12.766	13.381	13.490	NS	NS	NS
B16 (38.9 kDa)	16.113	16.271	16.575	16.391	NS	NS	NS
B17 (35.2 kDa)	11.439	10.637	8.743	10.533	NS	NS	NS
B18 (32.5 kDa)	14.065	13.109	14.398	12.235	NS	**	NS
B19 (31.1 kDa)	6.493	6.631	6.686	6.365	NS	NS	NS
B20-21[2] (30.3-29.0 kDa)	1.027	1.044	0.752	0.893	NS	NS	NS
B22 (27.7 kDa)	1.023	1.032	1.105	1.078	NS	NS	NS
B23 (26.3 kDa)	0.379	1.049	0.667	0.934	NS	*	NS
B24 (25.3 kDa)	7.968	7.139	7.907	7.111	NS	*	NS
B25 (24.2 kDa)	3.411	3.291	3.968	3.903	NS	NS	NS
B26 (20.5 kDa)	2.085	1.985	1.978	2.161	NS	NS	*

[1]Bands 10,11 and 12 co-migrate in some SDS-PAGE gels, then they were quantified together
[2]Bands 20 and 21 co-migrate in some SDS-PAGE gels, then they were quantified together

Table: Protein identification for the whole set of protein bands separated by SDS-PAGE acrilamide gels

Band [MWe[1]]	Identification	Accession no.[2]	MOWSE[3] scores	Sequence Coverage (%)	Matched Queries	MWt[4]
B1 (210.6 kDa)	Glycogen debranching enzyme [Bos Taurus]	gi|300794727	491	14	21	176.2
B2 (175.6 kDa)	Myosin-binding protein C, fast-type [Sus scrofa]	gi|335290041	111	14	13	128.4
B3 (145.1 kDa)	Muscle glycogen phosphorylase [Sus scrofa]	gi|300119711	243	32	24	97.6
B4 (114.1 kDa)	Muscle glycogen phosphorylase [Sus scrofa]	gi|106073338	688	41	27	84.4
B5 (97.8 kDa)	Glycogen phosphorylase, muscle form isoform 1 [Sus scrofa]	gi|335281566	707	35	25	97.7
B6 (86.8 kDa)	Muscle 6-phosphofructokinase [Sus scrofa]	gi|95117652	524	35	22	82.4
B7 (73.8 kDa)	heat shock 70kDa protein 8 [Sus scrofa]	gi|345441750	645	35	16	71.1
B8 (70.5 kDa)	Muscle glycogen phosphorylase [Sus scrofa]	gi|106073338	560	37	23	84.4
B9 (61.7 kDa)	Albumin, partial [Sus scrofa]	gi|164318	808	38	20	71.3
B10 (56.8 kDa)	Phosphoglucomutase-1 [Sus scrofa]	gi|350538593	721	37	17	62.0
B11 (54.7 kDa)	Pyruvate kinase isozymes M1/M2 “PK” [Sus scrofa]	gi|335292434	592	28	17	68.5
B12 (53.5 kDa)	Glucose-6-phosphate isomerase [Sus scrofa]	gi|47523720	509	25	15	63.1
B13 (49.9 kDa)	UTP-glucose-1-phosphate uridylyltransferase [Sus scrofa]	gi|47522786	218	36	12	57.1
B14 (48.2 kDa)	β-enolase “ENO3” [Sus scrofa]	gi|113205498	264	46	14	47.4
B15 (43.5 kDa)	β-enolase”ENO3” [Sus scrofa]	gi|113205498	786	66	22	47.4
B16 (38.9 kDa)	Creatine kinase M-type [Sus scrofa]	gi|184018722	688	40	12	43.3
B17 (35.2 kDa)	Fructose-bisphosphate aldolase A [Bos taurus]	gi|156120479	704	53	14	39.9
B18 (32.5 kDa)	Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) “GAPDH” (EC 1.2.1.12)- pig	gi|65987	767	55	14	35.9
B19 (31.1 kDa)	L-lactate dehydrogenase A chain [Sus scrofa]	gi|288860136	124	34	10	36.9
B20 (30.3 kDa)	creatine kinase M chain [Bos taurus]	gi|4838363	219	22	4	43.2
B21 (29.0 kDa)	Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) “GAPDH” (EC 1.2.1.12)- pig	gi|65987	403	45	9	35.9
B22 (27.7 kDa)	Creatine kinase M-type [Sus strofa]	gi|194018722	462	27	12	43.3
B23 (26.3 kDa)	β-enolase “ENO3” [Bos taurus]	gi|77736349	282	32	9	47.4
B24 (25.3 kDa)	Carbonic anhydrase 3 “CAIII” [Sus scrofa]	gi|56711366	858	76	21	29.7
	Phosphoglycerate mutase-2 “PGM2” [Sus scrofa]	gi|201066358	400	56	13	28.8
B25 (24.2 kDa)	Triosephosphate isomerase 1 [Sus scrofa]	gi|262263205	441	71	14	26.9
B26 (20.5 kDa)	Adenylate kinase isoenzyme 1 “AK-1” [Sus scrofa]	gi|350579686	539	65	15	21.7

[1] MWe is the experimental molecular weight (kDa)

[2]Accession number correspond to NCBInr database

[3] The MOWSE score is a numeric descriptor of the likelihood that the identification is correct. Protein scores greater than 69 are significant ($P < 0.05$)

[4] MWt is the theoretical molecular weight (kDa)