1 **TITLE:**

2 Effect of sex and RYR1 gene mutation on the muscle proteomic profile and main

3 physiological biomarkers in pigs at slaughter

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27 **ABSTRACT**

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

42

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

44

45 **1. Introduction**

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

51 The effect of gender on the pig carcass development has been described in the literature.
52 In general, males present less accumulation of fat tissues (Gispert et al., 2010), but there
53 is still controversy about the effect of gender on the incidence of pale, soft and
54 exudative (PSE) meat, with some studies showing significant effects (Cisneros, Ellis,
55 McKeith, McCaw, & Fernando, 1996; Channon, Kerr, & Walker, 2004) whereas others
56 did not (Blanchard, Warkup, Ellis, Willis, & Avery, 1999; Channon, Payne, & Warner,
57 2000). These differences between experiments could be due in part to different *ante*
58 *mortem* handling conditions, which may influence the animal stress reactions at
59 slaughter and could have consequences on the ultimate meat quality (Boler et al., 2010;
60 Hambrecht et al., 2005a,b; Terlouw and Rybarczyk, 2008; D'Eath et al., 2010).

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

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

69 on the genetic background, the tissue physiological milieu and the animal's perception
70 of danger or fear during the slaughter procedure.
71 To date, there is still no precise definition of animal stress, probably due to the
72 complexity of different physical and psychological stressful situations, although it can
73 be described as "the physiological, behavioural and psychological state of the animal
74 when confronted with, from the animal's point of view, a potentially threatening
75 situation" (Terlouw, 2005).
76 Furthermore, it is important to note that the stress level of the animal depends indirectly
77 on the situation and directly on the animal's evaluation of the situation (Terlouw, 2005).
78 For this reason, meat scientists show increasing interest in the identification of animal-
79 based biomarkers that could be indicators of stress at slaughter and even that could be
80 used as indirect predictors of the ultimate meat quality. In this field, proteomics is a
81 promising tool, although its application is still in its infancy and very few studies have
82 focused on stress-dependent muscle proteome changes (Franco et al., 2015; Oliván et
83 al., 2016). The objective of this work was to investigate the effect of gender
84 (Male/Female) and RYR1 genotype (NN/Nn) on physiological, biochemical and
85 proteomic variables detected in the carcass that might influence the process of meat
86 quality acquisition and reflect animal's susceptibility to stress at slaughter.
87

88 ## 2. Materials and Methods

89 This study was approved by the Institutional Animal Care and Use Committee (IACUC)
90 of IRTA (Monells, Spain). The care and use of animals were performed in accordance
91 with the European Union Directive 2010/63 on the protection of animals used for
92 experimental and other scientific purposes (EU, 2010).
93

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*

118 At the slaughterhouse, blood samples were collected at exsanguination from each pig in
119 10-mL tubes without anticoagulant. Serum were obtained by centrifugation at 2000× g
120 for 10 min and immediately frozen at −80 °C until analysis.
121
122 *2.3. Biochemical and physiological parameters*
123 Metabolites analyzed were:
124 - Markers for glucose utilization: glucose (Hexokinase method, Olympus System
125 Reagent OSR), lactate (Enzymatic method LOD -Lactate Oxidase-, Olympus System
126 Reagent OSR).
127 - Markers of nitrogen metabolism: creatinine (Jaffé method, Olympus System Reagent
128 OSR), urea (GLDH method, Olympus System Reagent OSR), total proteins (Biuret
129 method, Olympus System Reagent OSR).
130 - Lipid metabolism markers: triglycerides (GPO-PAP method, Olympus System
131 Reagent OSR), total cholesterol (CHOP-PAP method, Olympus System Reagent OSR),
132 HDL-cholesterol (HDL-chol, Immunoinhibition method, Olympus System Reagent
133 OSR), LDL-cholesterol (LDL-chol, Selective protection method, Olympus System
134 Reagent OSR), non-esterified fatty acids (NEFAs, NEFA-C reagent, Wako Chemicals)
135 and 3-hydroxybutyrate (BHB, Ranbut reagent, Randox Laboratories, Ltd).
136 - Acute phase proteins "APPs" as inflammatory markers: haptoglobin (Phase
137 Haptoglobin, Tridelta Ltd), C-reactive protein (CRP, immunoturbidimetric method,
138 Olympus Systems Reagent) and Pig-MAP (ELISA, PigChamp ProEuropa).
139 - Skeletal muscle marker: creatine kinase (CK, IFCC method, Olympus System Reagent
140 OSR).
141 - Oxidative stress marker: glutathione peroxidase GPx (Cumene Hydroperoxyde
142 method, Ransel, Randox Laboratories Ltd).

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

144 All parameters were determined by spectrophotometric techniques in the analyzer
145 Olympus AU400, with the exception of Pig-MAP and cortisol, which were determined
146 by ELISA.

147

148 *2.4. Carcass measurements and meat sampling*

149 The skin lesions in each pig were assessed using the Welfare Quality® protocol
150 (Dalmau, Temple, Rodríguez, Llonch, & Velarde, 2009) considering 5 regions (ears,
151 front, middle, hind-quarters and legs) in one side of the carcass after scalding. Values of
152 0 (<2 lesions in all regions), 1 (2-10 lesions in at least one region) and 2 (>10 lesions in
153 at least one region) were used by a trained observer at the slaughter line.

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

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

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

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

168 container (Meat juice collector, Sarstedt, Nümbrecht, Germany) during 24 hours at 4ºC,
169 obtaining the drip loss percentage by gravimetry, according to the method described by
170 Rasmussen and Andersson (1996).
171 Instrumental texture was determined in LT samples by using the Warner Bratzler test,
172 following the procedures described in Ampuero-Kragten and Gil (2015). Samples were
173 vacuum packaged at 24 h *post mortem* and stored at 4ºC, and they were frozen (-20ºC)
174 after 1, 3 and 5 d aging to allow muscle tenderization. Each sample was thawed
175 overnight at 4ºC, cooked in an oven until a core temperature of 71ºC, and then 5
176 subsamples were obtained by using a perforating punch. These subsamples were
177 individually analyzed for instrumental toughness (maximum shear force, in kg) with the
178 TA.XT plus Texture Analyzer (Stable Microsystems, Haslemere, UK) and the mean
179 value for each animal was calculated.
180
181 *2.5. Sarcoplasmic Protein Extraction and Electrophoresis*
182 The sarcoplasmic protein fraction, which contains most of the enzymes of the glycolytic
183 pathway and other metabolic proteins (Hollung et al., 2007), were extracted from each
184 individual muscle sample (one per animal), taken immediately after slaughter, and
185 quantified following the method described by Jia et al. (2009). A total of 600 mg of
186 muscle tissue was dissected and homogenized in 2 mL of Tris-EDTA-Sucrose "TES"
187 buffer (10 mM Tris [pH7.6], 1 mM EDTA, and 0.25 M sucrose), using a Polytron
188 PT1200 E (Kinematica Inc., Luzern, Switzerland) three times for 15 s at maximum
189 speed. The homogenate was centrifuged (30 min at 8,800 x *g*) at 4°C to remove TES-
190 insoluble proteins. Protein concentrations were measured with a commercial kit at 760
191 nm (RC DC Protein Assay, Bio-Rad Laboratories, Hercules, CA) in a

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

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

242 modifications were considered (Cys as S carbamidomethyl derivative and Met as
243 oxidized methionine); iv) one missed cleavage site was allowed; v) precursor tolerance
244 was 100 parts per million and MS/MS fragment tolerance was 0.3 Da; vi) peptide
245 charge: 1+; and vii) the algorithm was set to use trypsin as the enzyme. Protein
246 candidates produced by this combined peptide mass fingerprinting/tandem MS search
247 were considered valid when the global Mascot score was greater than 85 with a
248 significance level of $P < 0.05$.
249

250 *2.7. Statistical Analysis*

251 The effect of sex (M/F) and genotype (NN/Nn) on carcass and meat quality traits, blood
252 biochemical and muscle proteomic variables was analyzed by Analysis of Variance
253 (ANOVA) using the General Linear Model (GLM) procedure of SPSS (v 15.0 2006,
254 SPSS Inc, Chicago, USA). The model included sex, genotype and its interaction as
255 fixed factors and slaughter day (batch) as random factor. When the interaction was
256 significant, the differences between the four treatments (M-NN, M-Nn, F-NN, F-Nn)
257 were analysed by the Tukey post-hoc test. The *post mortem* evolution of meat toughness
258 (Warner Bratzler maximum shear force) was analysed by GLM including sex, genotype,
259 aging time and their interactions as fixed factors and animal as random factor. Bivariate
260 correlations were calculated using Pearson's correlation coefficient.
261 Furthermore, multivariate analysis (PCA) was performed in order to study the
262 relationships between meat quality and physiological, biochemical and proteomic
263 variables obtained for every animal studied, by using XLStat software (XLStat 2013,
264 Addinsoft Inc, Paris, France). The Kaiser-Meyer-Olkin test was performed in order to
265 measure sampling adequacy for each variable in the model, and only variables with

266 KMO over 0.6 were selected. The overall KMO measure of the performed PCA was
267 0.725.
268

269 **3. Results and discussion**

270 *3.1. Carcass and meat quality*

271 Female pigs showed lower muscle pH than entire males (Table 1), with significant
272 differences at 45 min *post mortem* (P = 0.002). This agrees with D'Souza and Mullan
273 (2002) and D'Eath et al. (2010), who found lower pH (P = 0.006) in the loin muscle of
274 females compared with castrated pigs. These differences may be in part due to
275 physiological and metabolic differences in the cell response, indicating in some extent
276 higher susceptibility of females to stress at slaughter. This is a controversial issue, as it
277 has been postulated that entire male pigs are more susceptible to stress, because they
278 show more aggressive behavior than females and castrates (Fàbrega et al., 2010), while
279 in cattle Tarrant (1990) showed that females and young animals are more susceptible to
280 stress compared to males and older animals.
281 When looking to the effect of genotype, we found that the *post mortem* muscle pH
282 decline was faster in animals heterozygous for the RYR1 mutation (Nn), that showed
283 significantly (P < 0.001) lower pH at 45 min *post mortem*, while the ultimate pH did not
284 differ between genotypes (Table 1), so the pH amplitude (45 min - 24 h) was lower in
285 the Nn group (0.76 vs 1.03 for Nn and NN, respectively, P < 0.05).
286 Furthermore, Nn animals produced meat with higher values of electrical conductivity
287 (EC) (P < 0.05) and also higher drip loss (P < 0.001). Thus, the pH amplitude correlated
288 negatively with EC (r=-0.702, P < 0.001) and drip loss (r=-0.726, P < 0.001) and EC
289 and drip loss showed a positive and significant correlation (r= 0.858, P < 0.001). These
290 differences seem to indicate *post mortem* muscle metabolic differences due to the RYR1

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).

315 Another key quality trait, such as meat toughness, was significantly affected by RYR1
316 genotype (P < 0.05), with Nn animals exhibiting tougher meat (higher shear force)
317 along the process of meat aging (1 to 5 d *post mortem*, see Fig. 1), which agrees with
318 previous reports that described higher shear force and less tender meat in Nn than in
319 halothane free (NN) pigs (Channon et al., 2000; Fernández et al., 2002; Van den
320 Maagdenberg, Stinckens, Lefaucheur, Buys, & De Smet, 2008).
321 Furthermore, our results indicate increasing differences of shear force between
322 genotypes as the process of meat tenderization progressed (Fig. 1), although the
323 interaction between RYR1 genotype and aging time was not significant (P = 0.737).
324 Obviously, meat tenderness tended to increase as aging time increased in all meat types,
325 but the effect of the RYR1 mutation on meat toughness is probably related to
326 differences in the *post mortem* metabolism. All data recorded in this work (faster pH
327 decline, higher drip loss) indicate a fast *post mortem* metabolism in the muscle of Nn
328 pigs, confirmed in previous works, such as the one by Cheah, Cheah, & Krausgrill
329 (1995), who observed higher sarcoplasmic levels of calcium *in vivo* in the muscle from
330 Nn animals in contrast with NN, possibly causing a faster than normal rate of *post*
331 *mortem* muscle glycolysis in these pigs. In the same way, Depreux et al. (2002)
332 described a higher proportion of glycolytic fibres in the muscle of Nn genotype than
333 NN, expecting a more rapid *post mortem* pH decrease. This could imply a higher rate of
334 exhaustion of enzymes implicated in meat tenderization and therefore shorter
335 tenderization process. This agrees with previous histological and histochemical
336 investigations that have revealed increased fibre diameter and increased glycolytic
337 metabolic potential in the LT muscle of pigs with the RYR1 mutation, due to higher
338 proportion of the fast twitch glycolytic fibre type and lower of the slow twitch oxidative
339 type (Fiedler et al., 1999).

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

365 2). Heterozygous (Nn) pigs showed higher serum CK activity, which suggests increased
366 muscle damage, and higher CRP concentration, that may indicate higher stress level and
367 subsequent inflammation. To date, some APPs have been proposed as indicators of
368 animal stress (Saco et al., 2003; Piñeiro et al., 2007a; Salamano et al., 2008; Marco-
369 Ramell et al., 2011; Marco-Ramell et al., 2016), although the effect of stress on their
370 serum concentration remains controversial, since it is difficult to distinguish it from the
371 effect of trauma or subclinical infections.
372 On the other side, the higher GPx activity in homozygous (NN), but especially in
373 females (there was significant S*G interaction, $P < 0.01$) suggests more potent
374 antioxidant defenses in females, probably due to estrogen influence (Fig. 2b).
375
376 *3.3. Muscle proteins*
377 A total of 26 protein bands (201 to 20 kDa) were differentiated by SDS-PAGE gels in
378 the muscle sarcoplasmic extracts, as shown in Figure 3, where band names are denoted
379 by S of "sarcoplasmic" protein, followed by a number (1 to 26).
380 Table 3 gives the identification of protein bands with differential expression between
381 treatments and Table 4 shows the effect of sex and genotype and its interaction on the
382 abundance of these proteins.
383 *3.3.1. Effect of sex*
384 Myosin-binding protein C fast type, "MyBP-C" was overrepresented ($P < 0.05$) in the
385 muscle of females. MyBP-C belongs to the myosin-binding protein C family, including
386 fast- and slow-type isoforms, each of which is a myosin-associated protein found in the
387 cross-bridge-bearing zone (C region) of sarcomeric A bands, where interaction between
388 the thick and thin filaments occurs. Both structural and regulatory roles have been

389 proposed for MyBP-C, as it may modulate muscle contraction (Oakley, Hambly, Curmi,
390 & Brown, 2004).
391 The presence of S6 (muscle-6-phosphofructokinase “PFK-M”) was also significantly
392 affected by gender, with lower values in the muscle of females. PFK-M is the main rate-
393 controlling enzyme of glycolysis, which catalyzes the transfer of a phosphoryl group
394 from ATP to fructose-6-phosphate to yield ADP and fructose-1,6-bisphosphate. This
395 enzyme is tightly regulated and responds to diverse molecules and signals by changing
396 its catalytic activity and behaviour and is one of the few examples in which inhibition
397 by the substrate occurs, as ATP may inhibit PFK at different levels, depending on the
398 tissue metabolic state (Sola-Penna, Da Silva, Coelho, Marinho-Carvalho, & Zancan,
399 2010). Furthermore, lactate potentiate the inhibitory effects of ATP on PFK (Leite, Da
400 Silva, Coelho, Zancan, & Sola-Penna, 2007). Then in our study underexpression of
401 PFK-M in the muscle of females, that showed faster *post mortem* acidification, could be
402 potentiated by inhibition due to lactate, although we can not discard a possible lower
403 inherent PFK-M concentration in the muscle of females due to physiological differences
404 between males and females.
405 *3.3.2. Effect of genotype*
406 Genotype affected the presence of four peptide bands, thus producing lower presence of
407 S2 (MyBP-C, $P < 0.05$), S18 (glyceraldehyde-3-phosphate dehydrogenase “GAPDH”, P
408 < 0.01) and S24 (containing two proteins: carbonic anhydrase “CAIII” and
409 phosphoglycerate mutase-2 “PGM2”, $P < 0.05$) and higher of S23 (ENO3) in the
410 muscle of Nn pigs (Table 4).
411 Changes of MyBP-C, which corresponds to the muscle fibre structure, could be due to
412 the above mentioned differences of fibre type composition between RYR1 genotypes,
413 while the other significant changes affected to metabolic enzymes (GAPDH, CAIII

414 and/or PGM2, ENO3) that showed significant correlation with the rate of *post mortem*
415 muscle pH decline (pH-amplitude), being this relationship positive for GAPDH
416 ($r=0.540$, $p < 0.01$) and CAIII/PGM2 ($r=0.410$, $P < 0.05$) and negative for ENO3 (r=-
417 0.541, $P < 0.006$). This agrees with results from Gagaoua et al. (2015) who found
418 negative relationship between ENO3 and pH decline in beef.
419 Lower GAPDH in the muscle of Nn pigs indicate lower glycolysis, which could be
420 produced by an earlier depletion of muscle metabolites (glycogen) due to stress, as
421 found by Fernandez et al. (2002). It is worthwhile to mention that GAPDH has recently
422 been implicated in different non-metabolic processes, including transcription activation
423 and initiation of apoptosis (Tarze et al., 2007). Moreover, GAPDH may act as a
424 reversible metabolic switch under oxidative stress (Agarwal et al., 2012).
425 With respect to ENO3, it is a glycolytic enzyme that has been associated in beef with a
426 faster *post mortem* muscle energy metabolism resulting in a faster pH decline (Gagaoua
427 et al., 2015), and also has been correlated to beef colour stability (Gagaoua et al., 2015;
428 Gagaoua, Terlouw, & Picard, 2017; Picard, Gagaoua, & Hollung, 2017) and to meat
429 tenderization (Lametsch et al., 2003; Polati et al., 2012). Furthermore, ENO3 has been
430 described as a hypoxic stress protein providing protection of cells by increasing
431 anaerobic metabolism (Pancholi, 2001; Wulff, Jokumsen, Højrup, & Jessen, 2012).
432 Then, it could be expected to find increased ENO3 in the muscle of pigs suffering
433 higher stress at slaughter, that is, those from the Nn genotype.
434 The interpretation of changes found in the protein band S24 become difficult due to the
435 co-migration of two proteins (CAIII and PGM2) and the resultant joint quantification,
436 which is one of the difficulties of using 1D electrophoresis for protein separation. Band
437 S24 showed significantly ($P<0.05$) lower abundance in the muscle extracts of Nn
438 animals, and this difference was consistent regardless of sex (males and females), but

439 we could not dilucidate if both proteins (CAIII and PGM2) or only one of them had
440 lower presence in Nn pigs. Anyway, lower CAIII (which functions as oxyradical
441 scavenger and thus protects cells from oxidative damage) could be expected in the
442 muscle of Nn pigs and would reflect lower level of the antioxidant defense, which
443 agrees with the findings of Laville et al. (2009) who described reduced abundance of
444 antioxidant proteins in the SM muscle of pigs with RYR1 mutation (nn genotype)
445 compared with NN pigs, probably because the nn muscle was less oxidative and in
446 consequence presented less antioxidative and repair capacities. The growing interest of
447 meat scientist for the role of the balance between oxidative stress and antioxidant
448 defense in the *post mortem* muscle is more than evident, and gives significant
449 correlations with ultimate meat quality traits such as meat colour and tenderness
450 (Laville et al., 2007, 2009; Jia et al., 2009; Ouali et al., 2013; Gagaoua et al., 2015,
451 2017; te Pas et al., 2017).

452 The other protein found in band S24 was PGM2, which catalyzes the interconversion of
453 2-phosphoglycerate and 3-phosphoglycerate in the glycolytic pathway and therefore it
454 has a role of regulation of the energy balance and in the glycogen metabolism and
455 glycolysis of the skeletal muscle (Fontanesi et al., 2008). This protein is encoded by a
456 gene localized on porcine chromosome 18 (Fontanesi, Davoli, Nanni Costa, Scotti, &
457 Russo, 2003) in a region where quantitative trait loci for drip loss, meat colour, fat
458 deposition, lean content, muscle fiber diameter and carcass quality have been identified,
459 and it has been described a significant association between PGM2 and drip loss in pigs
460 (Fontanesi et al., 2003), so a lower PGM2 abundance in the muscle of Nn pigs, that
461 showed higher drip loss, would be expected.

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

464 glycolysis function and a higher defense of the muscle cell to oxidative stress, which
465 could be related to metabolic changes due to the RYR1 mutation, that causes a
466 dysregulation of the calcium homeostasis and lead to neuromuscular disorders (Treves
467 et al, 2005) and even can affect immunological and neuroendocrine response of pigs to
468 stress (Ciepielewski et al., 2016). In our study we could not quantify changes in the
469 abundance of muscle proteins involved in calcium homeostasis, such as sarcalumenin or
470 calsequestrin-1, that were not separated in the 1D SDS-PAGE gels. These proteins have
471 recently been detected by 2D-electrophoresis in mice muscle by Picard et al. (2016)
472 who found that its abundance in the *Tibialis anterior* muscle (fast glycolytic) increased
473 in the absence of Hsp27 (heat shock protein that has been described as beef tenderness
474 biomarker, by the group of Picard).
475
476 *3.3.3. Interaction of sex and genotype*
477 The statistical analysis showed that there was a significant interaction between sex and
478 genotype for two muscle proteins: S9 (albumin) and S26 (AK-1). That is, in this study,
479 males tended to show higher muscle albumin expression than females, which could
480 reflect physiological differences related to the function of albumin in the skeletal
481 muscle, where it serves as a temporary amino acid storage site, maintains osmotic
482 pressure and acts as a transporter for free fatty acids (Ellmerer et al., 2000), but the
483 presence of the RYR1 mutation increased the albumin expression in the muscle of
484 females and decreased it in males. Regarding AK-1, which catalyzes the reversible
485 transfer of the terminal phosphate group between ATP and AMP and is a key enzyme in
486 the muscle energetic homeostasis, it showed slightly higher level in Nn genotypes,
487 which was more evident in males than in females, then showing significant interaction
488 between sex and genotype. Our results show lower AK-1 level in Nn females, which

489 could be related with alterations of the muscle homeostasis as a result of higher stress
490 response at slaughter, which is in agreement with a previous report from our group that
491 showed that higher pre-slaughter stress produced lower presence of AK-1 in the *post*
492 *mortem* muscle in pigs when mixed with unfamiliar animals (Oliván et al., 2016).
493
494 *3.4. Multivariate analysis*
495 Multivariate analysis was applied in order to obtain a synthetic assessment of the
496 complex relationships between the variables best suited for factor analysis (KMO>0.6),
497 that were: three meat quality traits (pH45, EC and drip), four serum metabolites
498 (glucose, creatinine, CK and GPx) and three protein bands containing muscle proteins
499 of the energy metabolism and antioxidant defense (GAPDH, ENO3 and CAIII/PGM2).
500 The biplot obtained via PCA (Fig. 4) showed that PC1 and PC2 explained 62 % of the
501 variability in the data.
502 The first principal component (PC1) distinguished in the positive side main variables
503 related to poor meat quality: drip loss, EC and meat toughness (shear force at 5 days
504 aging (WBSF-5d). Other variables with high loadings for PC1 were serum creatinine
505 and CK, which indicate higher muscle damage at slaughter, and ENO3, a muscle
506 protein that has been related to faster energy metabolism and faster pH decline in beef
507 (Gagaoua et al., 2015) and also to hypoxic stress (Sedoris et al., 2010).
508 Furthermore, carcass temperature showed a positive correlation with PC1, which all
509 together clearly indicates that the positive side of PC1 merged variables related to stress
510 at slaughter. These characteristics corresponded to animals of the Nn genotype, mainly
511 females, whose mean score showed high positive correlation to PC1 (Figure 4). By
512 contrast, the negative side of the PC1 grouped meat variables indicating normal *post*
513 *mortem* pH decline (higher pH at 45min), normal muscle glycolytic metabolism (higher

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

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

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

530 Furthermore, from a proteomic perspective, these results allowed the identification of
531 key proteins involved in the *post mortem* glycolytic pathway (GAPDH, PGM2, ENO3)
532 and the antioxidant defense (CAIII) of the muscle that contribute to the process of meat
533 quality acquisition and are influenced by pre-slaughter stress. These proteins have a
534 relevant role in the *post mortem* muscle metabolism and most of them have already
535 been identified as biomarkers of meat quality and animal stress (Laville et al., 2007,
536 2009; Guillemin, Bonnet, Jurie, & Picard, 2011; Gagaoua et al., 2015, 2017; Oliván et
537 al., 2016).

538 These results contribute to progress towards the comprehensive identification of
539 proteins linked to the process of meat quality acquisition, being ultimately modulated by
540 the animal's stress reaction at slaughter. Knowing the biological mechanism underlying
541 this process opens up the possibility of monitoring and predicting the resulting changes.
542 Once this is known, these potential protein biomarkers must follow a process of
543 evaluation and validation (Naylor, 2003; Te Pas, Hoekman & Smits, 2011; Picard &
544 Gagaoua, 2017), so further research is needed on a larger data set.
545

546 **4. Conclusions**

547 Pork quality development is largely governed by the rate and extent of *post mortem*
548 muscle metabolism, which is affected by animal factors like sex and RYR1 genotype,
549 with influence as well in the modulation of the animal's individual susceptibility to pre-
550 slaughter stress.
551 The results of this study showed that the sex and the RYR1 genotype affected several
552 blood biochemical parameters at slaughter and some muscle enzymes with key role on
553 the subsequent process of muscle-to-meat conversion, showing Nn females more
554 susceptibility to stress, with detrimental effect on meat quality.
555 These differences may be monitored by protein biomarkers related to the fibre
556 composition, the *post mortem* glycolytic pathway and the antioxidant defense of the
557 muscle. However, it is worthwhile to mention that the complex nature of the processes
558 that underlie the *post mortem* meat quality development and the high diversity of factors
559 that may influence the animal's susceptibility to stress at slaughter makes difficult to
560 find universal biomarkers. Then, more research is needed in order to apply combined
561 "omics" techniques that allow the identification of key protein biomarkers and to
562 validate them in different breeds and management systems.

563

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572

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892

893 Table 1 Least squares means and the effect of sex (S) and RYR1 genotype (G) and its

894 interaction (S*G) on carcass and meat quality traits.

895

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

896 Means in the same row followed by different superscripts are significantly different at $P < 0.05$.

897 [1]SEM: standard error of means

898 [2]EC: electrical conductivity

899 [3]WBSF: Warner-Braztler shear force at 1, 3 or 5 days *post mortem*

900

901

902 Table 2. Least squares means and the effect of sex (S) and RYR1 genotype (G) and its

903 interaction (S*G) on serum biochemical variables at slaughter.

904

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

905 Means in the same row followed by different superscripts are significantly different at $P < 0.05$.
906 [1]SEM: standard error of means
907 [2] HDL-chol: High density lipoproteins-cholesterol
908 [3] LDL-chol: High density lipoproteins-cholesterol
909 [4] NEFAs: non-esterified fatty acids
910 [5] BHB: β-hydroxybutyrate
911 [6] CRP: C-reactive protein
912 [7] CK: creatine kinase
913 [8] GPx: glutathione peroxidase

914

915
916 Table 3: Protein identification in noticeable bands of sarcoplasmic extracts separated by
917 SDS-PAGE acrilamide gels
918

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

919 [1]MWe is the experimental molecular weight (kDa)
920 [2]Accession number correspond to NCBInr database
921 [3]The MOWSE score is a numeric descriptor of the likelihood that the identification is correct. Protein scores greater
922 than 69 are significant ($P < 0.05$)
923 [4] MWt is the theoretical molecular weight (kDa)
924

925

926
927 Table 4. Least squares means and the effect of sex (S), RYR1 genotype (G) and its
928 interaction (S*G) on the expression of noticeable proteins of the muscle sarcoplasmic
929 extracts (optical density, in arbitrary units).
930
931
932

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

933 Means in the same row followed by different superscripts are significantly different at $P < 0.05$.
934 [1]SEM: standard error of means
935
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937

938 **Figure captions:**

939

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

943

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

946

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

950

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

953

954

955 Figure 1.

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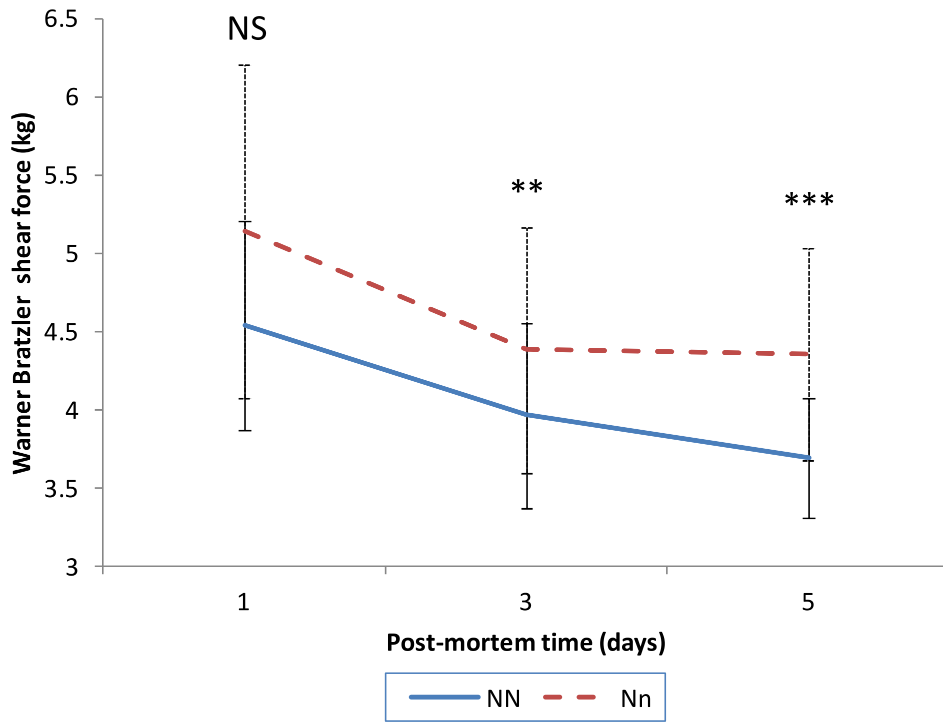

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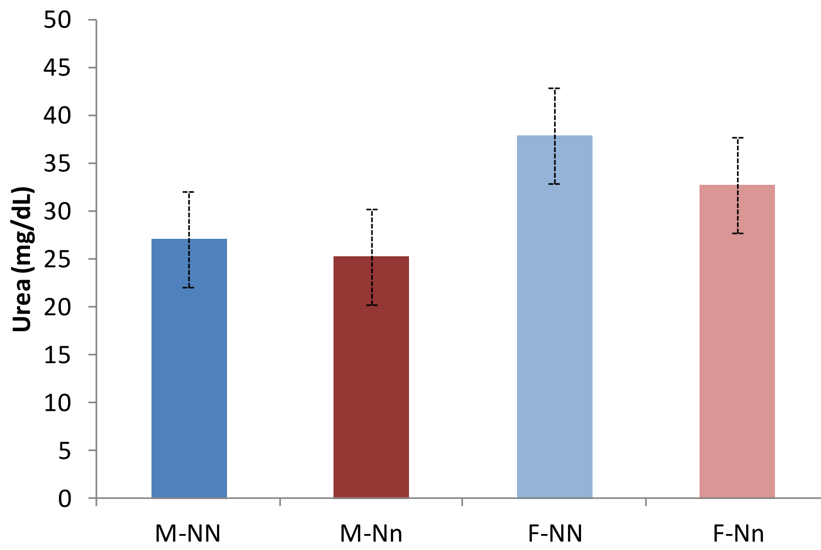
960

961 Figure 2.

962 a)



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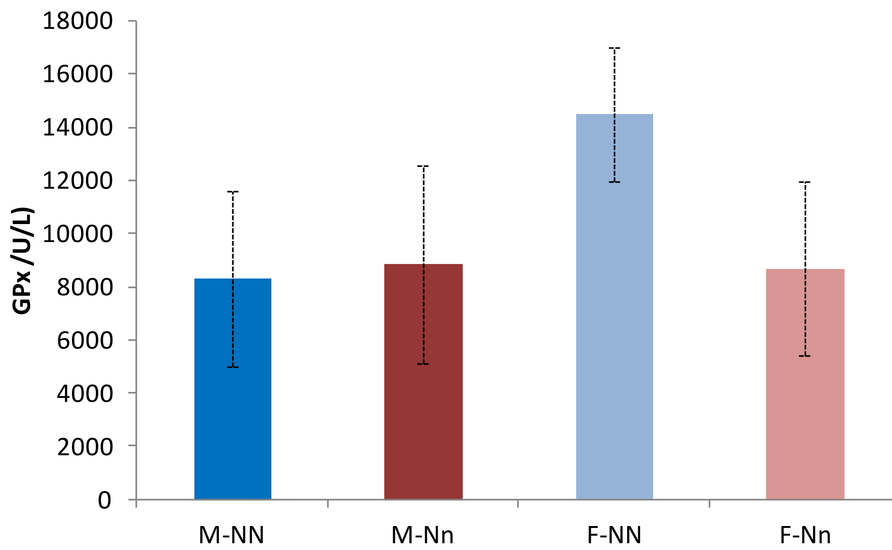
964 b)



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967 Figure 3.

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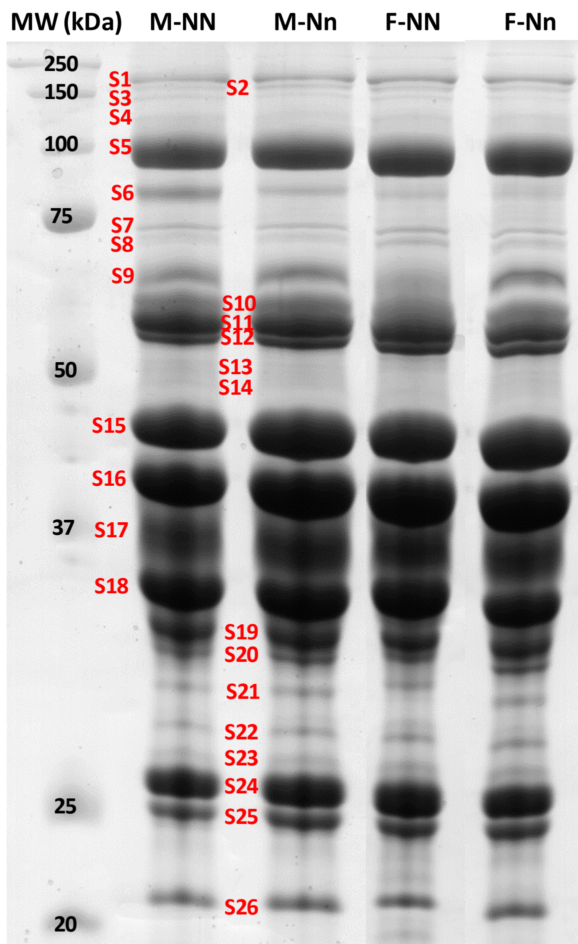

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971 Figure 4.

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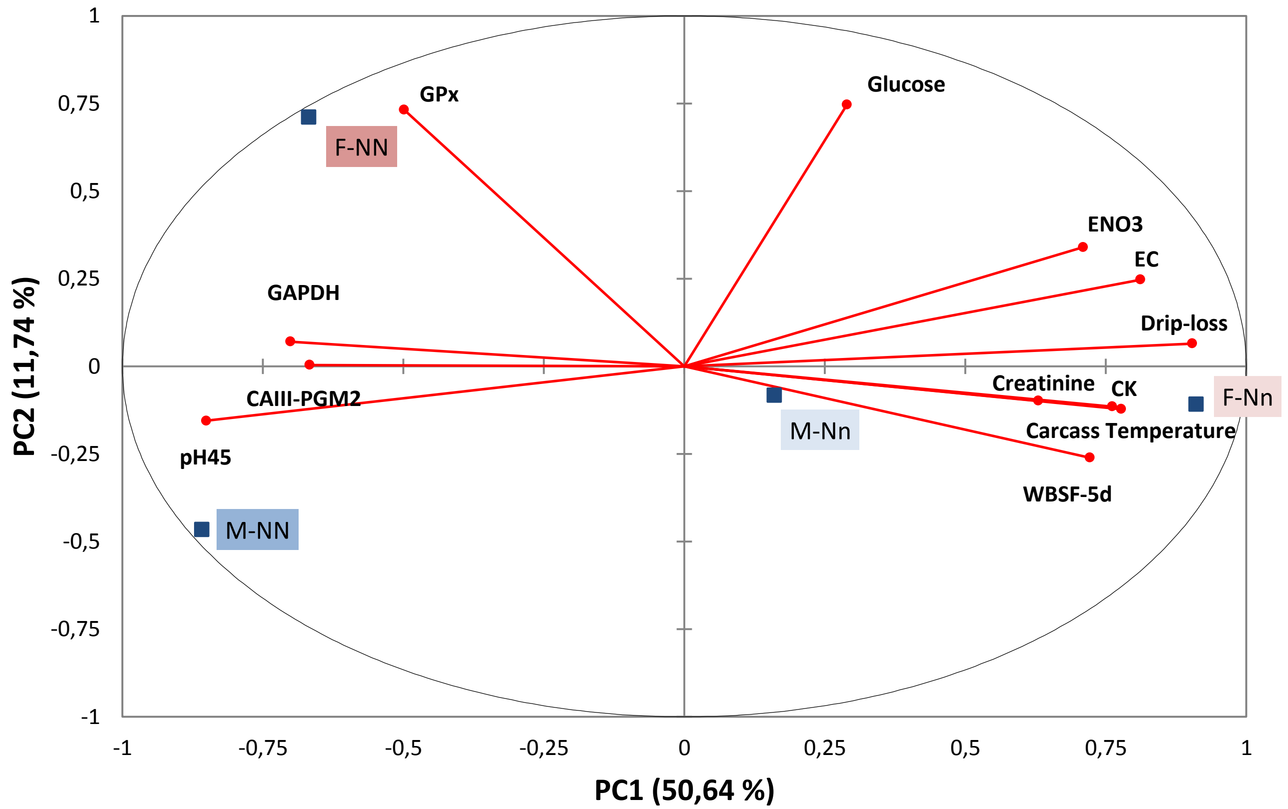

974

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