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PAPER

Genetic and environmental effects on a meat spotting defect in seasoned dry-cured ham

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Abstract

Purpose of this investigation was to determine the nature of a visible spotting defect on the slice of dry-cured ham and assess environmental and genetic causes of this frequent problem. A group of 233 pigs from commercial cross-breeding lines, progeny of ten boars and forty seven sows, was raised in a single herd to obtain the Italian Heavy Pig, typically slaughtered at 160±10 kg live weight and older than 9 months of age. A quality evaluation of their right dry-cured hams, seasoned according to the Parma P.D.O. protocol, was undertaken. Each ham was cross-sectioned to obtain a slice of *Semimembranosus*, *Semitendinosus* and B*iceps Femoris* muscles. The focused phenotype was the presence/absence of brownish spots in these muscles, which represent a remarkable meat defect with strong impact on the final sale price. Environmental and management factors were considered in order to evaluate variability related to the phenotype. Animals were raised on two different flooring types (concrete and slatted floor) and a Vitamin C diet was also supplemented in the last 45 days before slaughtering to half of the animals. While the pre-planned environmental effects did not show any significant contribution to the total variability of the phenotype, the genetic analysis showed a near to zero value for heritability with a consistent 0.32 repeatability. The proportion of the total phenotypic variance was explained by an important dominance genetic component (0.26) indicating that the technological seasoning process may play a secondary role on the expression of this phenotype.

Introduction

Heavy pig breeding has an important economic role in the Italian swine agrifood industrial system. This particular kind of animal is geared towards the production of dry-cured ham obtained from their legs (Russo, 1989; Chizzolini, 1991). The total number of dry-cured hams produced under a PDO (Protected Designation of Origin) protocol is around 12.5 millions of pieces per year (Istituto Nord Est Qualità – Istituto Parma Qualità, 2007). In particular, pigs, raised for Parma ham production, are slaughtered older than 9 months of age at a live weight of 160±10 kg. In this way a pig reaches a more mature carcass conformation in order to obtain an optimal balance between the fat (fat cover thickness and intramuscular fat) and the lean compounds that represent the most important components of a good raw material (Bellatti *et al*., 1996). Selected legs are then salted and seasoned for twelve months at least in order to produce the final high quality dry-cured ham. The typical Italian PDO dry-cured ham requires therefore an acceptable level of meat hardness, fat covering (not less then 20 mm of thickness), marbling (intramuscular fat) and a bright red colour (Parma Ham Consortium, 2007; Russo, 1991; Chizzolini *et al*., 1996). A defect detected in one of these components leads to a significant economic loss, since over 40% of the value of a heavy pig is represented by its two legs (Centro Ricerche Produzioni Animali, 2005).

Before being marketed, each single dry-cured ham is inspected and in case approved by the Parma PDO Consortium by the official fire branding (five-pointed Ducal Crown) on the ham's skin. After this approval, part of the hams can be sold in pieces or in pre-sliced packages and some defects can now appear. In recent years there is a growing concern about an apparently increasing presence of brownish spots in the *Semimembranosus*, *Semitendinosus* and *Biceps Femoris* muscles in correspondence with major blood vessels. Normally, this defect does not originate from microbial spoilage, as confirmed by regular microbial counts usually found in defective hams. Rather, stunning methods have been reported as the main cause of these small spots spreading within the muscles (Parolari, 1996). Obviously, this defect can have a large impact on the market price of the ham.

Vitamin C is a powerful biological antioxidant (Benzie, 1996). In general, pigs synthesize vitamin C and according to the National Research Council (1998) do not require it in the basal diet for normal growth. However it could be possible that endogenous synthesis is inadequate to maximize vitamin C contribution to oxidative stability during periods of adverse environmental conditions, disease, or exposure to stressors. In such situations endothelial cells could be less protected and capillary permeability increases, which may favour the development of haemorrhagic patches in muscle. Effects of vitamin C supplementation on pork meat quality may be associated with altered glucose and glycogen metabolism (Mourot *et al*., 1992). The ascorbic acid metabolite, oxalic acid, has been shown to function as a glycolytic inhibitor, which reduces lactic acid production resulting in a higher early post-mortem pH and reducing water loss from the muscle (Kremer *et al*., 1999). Vitamin C has further been reported to decrease the severity of a pre-slaughter stress response by inhibiting glucocorticoid synthesis (Pettigrew and Esnaola, 2001).

Provision of dietary vitamin C to animals is difficult because the vitamin is very unstable in feed. Vitamin C can be easily supplemented through drinking water or liquid feed for short periods of time at the critical pre-slaughter stage. In addition, vitamin C is rapidly excreted when the plasma concentration exceeds the renal threshold level and the half-life decreases as consumption increases (Tsao, 1997).

Regarding the housing system, with special attention to flooring types, a remarkable list of experimental results have been focused on

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growing performance, behaviour, carcass traits, fresh meat quality and leg joint disorders (Gentry *et al*., 2002; 2004; Candotti *et al*., 2004; Lebret *et al*., 2006). Considering the different mobility allowed to a pig raised on concrete or slatted floor and its impact on a variety of different phenotypes we wondered if a possible reason of our meat spotting defect could be associated to the different use of leg muscles in these two flooring conditions. Purpose of this paper was first to investigate the real nature of these spots both from a chemical and histological point of view. Secondly, we tried to define some possible environmental factors affecting this phenotype. In particular, we wondered if the two mostly used type of flooring (slatted and concrete floor), affecting the locomotion of the animals, could impact on muscle quality. Another point was related to the possible protecting action of Vitamin C on the blood vessel wall. Finally we investigated about the impact of genetics on the expression of this undesired phenotype, trying to assess its parent-offspring transmission and its repeatability in different muscles for possible selection programmes.

Materials and methods

Animals

Despite the original numbers involved in the experiment were larger, we report here the numbers of the final data-set on which all the analyses were performed. Two hundred and thirty three pigs (115 females and 118 barrows) from 47 sows and 10 boars, from commercial cross-breeding lines, were raised in the same environmental and management condition. At weaning the animals were split in two different sex-balanced experimental groups raised on two different flooring types: slatted and concrete floor. The splitting was made choosing, within each litter, pairs of piglets of the same sex and size and assigning them to the two different floor types. Half animals on slatted floor and half on the concrete floor were supplemented with vitamin C through liquid feed (150 IU/kg) above the standard normal requirement for growing pigs (National Research Council, 1998) in the last 45 days of their life cycle according to Table 1. At the end of their life cycle the animals were slaughtered, in the same slaughterhouse, in four different shifts during September 2005.

Dry-cured ham preparation and evaluation

Two hundred and thirty three right legs were subjected to the seasoning process for Parma dry-cured ham production. The major steps of the Parma ham production are here summarized according to the protocol of the Parma Ham Consortium (2007).

1. First salting (1-4ºC, 80% RH, 7 days);
2. Second salting (1-4ºC, 80% RH, 15/18 days);
3. Resting (1-5ºC, 75% RH, 60/90 days);
4. Seasoning period (eight months / controlled thermo-hygrometric conditions).

At the end of the seasoning process, dry-cured hams were boned and cross-sectioned. Hams were evaluated by a group of experts in order to detect anomalous properties. A ham slice, from the cross-section (Figure 1 A), was evaluated to detect and quantify the presence of brownish spots on *Semimembranosus* (SM), *Semitendinosus* (ST) and *Biceps Femoris* (BF) muscle surface (Figure 1 B). For each section, spot position (muscle) and size were detected and evaluated by a linear additive score: small (score=1), medium (score=2) and large spots (score=3). The slice was then vacuum packed for further biochemical and histological analysis.

Table 1. Animal distribution among sex, floor type and vitamin supplement.

	Slatted floor		Concrete floor		Total
Vitamin C	Yes	No	Yes	No	
Barrow	28	30	31	29	118
Female	22	27	34	32	115
Total	50	57	65	61	233

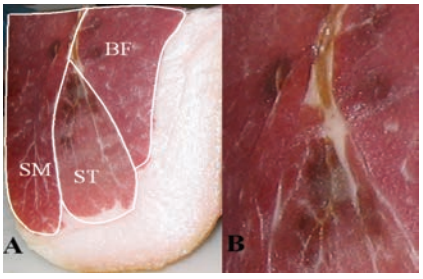

Figure 1. Dry cured ham cross-section (A) and meat spotting (B) in *Semitendinosus*, *Semimembranosus* and *Biceps Femoris* muscles.

Histology

For histology a random sampling of 55 muscle areas was analyzed. Small fragments (1.5 cm^3) of dry cured hams were excised from both the anomalous brown-spotted and normal red zones, and were fixed by immersion in neutral buffered formalin during 48 h. After the usual treatment for the paraffin embedding of the specimens, serial sections (3 μm-thick) were obtained by a microtome, and were subsequently stained with the haematoxylin-eosin sequential staining. The sections were observed utilizing an Olympus BX51 light microscope by an observer, who was not aware of the origin of the sections.

Biochemistry

The oxidative rancidity in spotted tissue, measured by the determination of free malondialdehyde, showed no differences when compared to the normal tissue. Therefore, after that defects caused from lipid oxidation was excluded, twenty-four samples from brown-spotted areas randomly chosen among different muscles of defective hams were sampled to evaluate the presence of haemoglobin. The separation of heme proteins was performed by HPLC using a hydrophobic interaction column according to the method of Oellingrath *et al*. (1990). Absorbance was measured at 280 nm (total protein profile) and at 420 nm (hemo protein profile) with a photodiode detector. Muscle samples were trimmed and homogenized using an Ultra Turrax with 30 mL of 20 mM trishydroxymethylamino-methane buffer, pH 8.5, 0.5 M NaCl, and centrifuged at 25,000 g for 30 min at 4°C. The supernatant was filtered through 0.22 μm microfilters before analysis. Horse skeletal myoglobin and porcine hemoglobin were used as standards.

Statistical analysis

The meat spotting scores on the complete data set (233 hams by 3 muscles = 699 observations) were analyzed by two different mixed models. The two models included the same fixed effects, but differed for the random effects in order to achieve the most complete interpretation of the genetic determination of the phenotype. The first model was the following:

$$y_{ijklmno} = \mu + SLD_i + MUS_j + FL_k + VIT_l + SEX_m + b_1X_1 + b_2X_2 + a_n + p_n + e_{ijklmno}$$

where:

$y_{ijklmno}$ = meat spotting score

μ = overall mean

SLD_i = fixed effect of slaughtering day (i=1, … 4)

MUS_j = fixed effect of the muscle (j = 1, … 3: SM, ST, BF)

FL_k = fixed effect of flooring type (k = 1, … 2: slatted or concrete floor)

VIT_l = fixed effect of vitamin supplementation

(l = 1, … 2: yes or not)
SEX_m = fixed effect o sex (m = 1, … 2: barrow or female)
X_1 = Age at slaughtering in days (covariate)
X_2 = Carcass weight in kg (covariate)
a_n = random additive animal effect (233 animals)
p_n = random non additive and permanent environmental effect
$e_{ijklmno}$ = random residual effect

Animal effects were split into two different sets of equations. For the first set a complete additive relationship matrix of the 233 animals was included. Solutions to these equations estimate the individual additive genetic merit (A) while the variance of this factor (σ^2_a) estimates the additive genetic variance (σ^2_A). The second set of equations takes into account any permanent effect, which corresponds to the sum of dominance (D), genetic interaction (I) and permanent environmental effect (PE) of each animal. Therefore the variance of this factor (σ^2_p) estimates $\sigma^2_D+\sigma^2_I+\sigma^2_{PE}$. The random residual effect can be equated in terms of genetic model to the temporary environmental effect (TE) with variance $\sigma^2_e = \sigma^2_{TE}$ (Van Vleck *et al*., 1987). Additive and permanent variance components were estimated by a MTDF Reml procedure (Boldman *et al*., 1995). From the three variance components, estimates of the total phenotypic variance ($\sigma^2_P= \sigma^2_a+\sigma^2_p+\sigma^2_e$), heritability ($h^2$) and repeatability (r) were computed as: $h^2=\sigma^2_a/\sigma^2_P$ and $r=(\sigma^2_a+\sigma^2_p)/\sigma^2_P$.

The same animal model was applied separately to the three muscles in order to detect genetic differences among the three phenotypes. In this case the permanent effect was excluded since one observation per animal was available. By this model only the additive component was therefore computed.

In the second model the random part was set up as a sib analysis (Falconer, 1989). A boar effect and a sow within boar effect were included, in addition to a residual within progeny error term. The equivalence of the statistical variance components to the genetic variances is reported in Table 2.

The between sow variance should include a common environmental component shared by full sib animals during the first three weeks of life only. The remaining environmental variance arises from causes of difference that are uncorrelated with whether the animals are related or not. Given the nature of the phenotype, which is measured after a full year of seasoning on hams of animals slaughtered at ten months of age, we assumed the common environmental component as equal to zero. From the three genetic variances, estimated by the preceding equations, the proportion of additive (h^2) and dominance variance (d^2) on the total phenotypic variance ($\sigma^2_P=\sigma^2_A+\sigma^2_D+\sigma^2_E$) was computed.

Results and discussion

Frequencies, means and environmental effects

Frequency of meat spotting presence/absence was recorded. One hundred and seventeen hams were spotted to various degrees while 116 were not spotted. Table 3 shows the distribution and frequencies of phenotypic scores for the three muscles. The maximum spotting score given was 10. Table 4 shows general statistics for covariates included in the two models.

Results of the analysis of variance of the fixed factors are shown in Table 5. Only slaughtering day and muscle location show significant effect on the meat spotting score. The least square means for the muscle effects were the greatest for ST (1.006), followed by SM (0.881) and BF (0.426). The only significant differences were between BF and SM (P=0.0004;) and BF and ST (P<0.0001). The other fixed effects, housing surface (slatted/concrete floor), vitamin C supplementation, sex, carcass weight and age at slaughtering did not influence the meat spotting score.

Histology

The histological examinations of sections coming from both the anomalous brown-spotted and normal red zones showed a quite completely conserved structure of the muscle tissue and the accompanying adipose tissue. The observed muscle structure appeared not modified in the anomalous brown-spotted zones.

Biochemistry

Amounts of myoglobin and haemoglobin standards in the range of 1-10 μg were subjected to HPLC analysis to establish calibration curves and peaks were used to identify heme proteins in the samples. The presence of haemoglobin in tissue extract were detected in all samples analysed. Using the method of Oellingrath *et al*. (1990), the amount of haemoglobin was estimated ranging from 50 to 450 μg g^{-1} tissue, depending on the size of the brown-spots in the tissue analysed. The nature of the spots seems therefore to depend on the presence of haemoglobin in the tissue.

Genetic analysis

Table 6 reports the genetic variances computed from the estimated components in the animal and sib model.

The additive genetic variance component, V(A), was very low. The heritability was virtually zero and this result was confirmed by the separate analyses of the three muscles, with the exception of the *Biceps Femoris* for which a heritability estimate of 0.09 was obtained. On the opposite, the permanent environmental variance was much higher, resulting in a

Table 2. Equivalence between genetic and statistical variances.

	σ^2_A	σ^2_D	σ^2_E
σ^2Boar	¼	0	0
σ^2Sow (boar)	¼	¼	0
σ^2Within	½	¾	1

Table 3. Spotting data layout.

Muscle	Not spotted hams	Spotted hams	Small spots Score = 1	Medium spots Score = 2	Large spots Score = 3
SM	152	81	36	38	20
ST	152	81	32	41	30
BF	188	45	15	17	15

SM, *semimembranosus* muscle; ST, *semitendinosus* muscle; BF, *biceps femoris* muscle.

Table 4. Statistics for covariates.

Covariate	Mean	SD	Min	Max
Age, d	301.4	10.7	284	331
Weight, kg	142.3	16.1	97.5	183.4

SD, standard deviation.

Table 5. Type 3 test for fixed effects.

Fixed factors	F value	P
Vitamin C	0.05	0.8294
Slaughtering day	15.21	<0.0001
Sex	2.97	0.0851
Flooring type	2.04	0,1537
Muscle	11.47	<0.0001
Carcass weight	0.55	0.4576
Age at slaughtering	0.73	0.3921

Table 6. Variance components analysis and genetic parameters computed from animal model for each single muscle and together (full animal model), and from sib analysis.

Model	V(A)	V(D)	V(I)	V(PE)	V(TE)	V(P)	h^2	r	d^2
SM	0.00001		-		1.9637	1.9637	0.00	-	-
ST	0.00008		-		2.9158	2.9159	0.00	-	-
BF	0.08715		-		0.8319	0.9191	0.09	-	-
Full animal model	0.00020		0.6178		1.3157	1.9336	0.00	0.32	-
Sib analysis	0.00005	0.5204		1.4991		2.0195	0.00	-	0.26

V(A), additive genetic variance; V(D), dominance variance; V(I), interaction variance; V(PE), permanent environment variance; V(TE), temporary environment variance; V(P), phenotypic variance; SM, *Semimembranosus* muscle; ST, *Semitendinosus* muscle; BF, *Biceps Femoris* muscle.

repeatability estimate of 0.32. From the sib analysis it was possible to obtain a separate estimate of the dominance variance, V(D), while the other variances, V(I) + V(PE) + V(TE), were pooled in a single estimate. From this model we have therefore estimated the d^2 parameter (0.26). Pooling together the results from the two models it is possible to deduce a p^2 parameter (0.06) that corresponds to the ratio of the interaction and permanent variance components over the total phenotypic variance.

The observed phenotype seems therefore under a minimal genetic control if we focus on the additive effect, i.e. the heritable effect. A much more important role is played by the dominance genetic effect which corresponds to the d^2=0.26 parameter. This part of the total variability indicates that genetics has a primary responsibility in the undesired expression of this phenotype even if the possibility to select against the meat spotting is virtually zero. Another fraction of the total variance (p^2 =0.06) can be assigned to permanent effects associated to the technological processing of the legs, from the exsanguination to the massaging of the hams through all the salting and seasoning process. Finally, besides these repeatable fractions of the total variance (r= 0.32), there is a large random environmental variability not yet assignable to systematic factors.

Conclusions

Certainly meat spotting is a remarkable problem involving dry-cured ham quality considering that an important economic loss is referred to this undesirable defect. In fact its market price is greatly dependent on its aspect that can induce the final customer to refuse the purchase of spotted slices. Our results show that some herd management conditions and technological seasoning process do not significantly influence meat spotting. Besides, the histological analysis show that the described defect do not have structural bases since muscle fibres and adipose tissue conserve their usual aspect in both the anomalous brown-spotted and normal red zones. Moreover, considering the hemorrhagic nature of this phenomenon the possible protecting action of vitamin C on the blood vessel did not show any significant effect on the phenotype expression.

The genetic analysis indicates that to select against this defect is quite difficult or even impossible, as its heritability is virtually zero. The estimated repeatability was 0.32, which we were able to split in two different parts. The p^2 parameter (0.06) indicates that only a small part of the total variability can be assigned to the permanent environmental effects associated to the technological process. On the contrary the d^2 parameter (0.26) indicates the important role on the phenotype expression played by the dominance genetic component. So it seems reasonable to hypothesize that the increasing appearance of this defect can be referred to a corresponding increasing number of available commercial hybrids and intra-breed lines used to produce home made terminal crosses. Undesirable genetic combinations can therefore circulate in the heavy pig populations maybe selected because of some favourable phenotypic trait. Unfortunately the undesired effect of such combinations can be detected only one year after slaughtering when tracing the genetic origin of the spotted hams is almost impossible.

Further investigations would be of course necessary to validate this hypothesis and in case identify the better possible crosses.

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