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Genome-wide association and pathway analysis of carcass and meat quality traits in Piemontese young bulls

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*A key concern in beef production is how to improve carcass and meat quality traits. Identifying the genomic regions and biological pathways that contribute to explaining variability in these traits is of great importance for selection purposes. In this study, genome wide-association (**GWAS**) and pathway-based analyses of carcass traits (age at slaughter (**AS**), carcass weight (**CW**), carcass daily gain (**CDG**), conformation score and rib-eye muscle area) and meat quality traits (pH, Warner-Bratzler shear force, purge loss, cooking loss and colour parameters (lightness, redness, yellowness, chroma, hue)) were conducted using genotype data from the 'GeneSeek Genomic Profiler Bovine LD' array in a cohort of 1166 double-muscled Piemontese beef cattle. The genome wide-association analysis was based on the GRAMMAR-GC approach and identified 37 significant single nucleotide polymorphisms (**SNPs**), which were associated with 12 traits ($P<5\times10^{-5}$). In particular, 14 SNPs associated with CW, CDG and AS were located at 38.57 to 38.94 Mb on* Bos taurus *autosome 6 and mapped within four genes, that is, Leucine Aminopeptidase 3, Family with Sequence Similarity 184 Member B, Non-SMC Condensin I Complex Subunit G and Ligand-Dependent Nuclear Receptor Corepressor-Like. Strong pairwise linkage disequilibrium was found in this region. For meat quality traits, most associations were 1 SNP per trait, except for a signal on BTA25 (at ~11.96 Mb), which was significant for four of the five meat colour parameters assessed. Gene-set enrichment analyses yielded significant results for six traits (right-sided hypergeometric test, false discovery rate <0.05). In particular, several pathways related to transmembrane transport (i.e., oxygen, calcium, ion and cation) were overrepresented for meat colour parameters. The results obtained provide useful information for genomic selection for beef production and quality in the Piemontese breed.*

Keywords: genomic analysis, functional analysis, beef, double-muscled, Piedmontese

Implications

Meat quality attributes influence consumer acceptance and purchase intention. In this study, we investigated the genetic basis of carcass and meat quality traits in double-muscled Piemontese cattle breed, which is known to be characterised by large muscle growth and low fat deposition. Besides the role of myostatin mutation, which is almost fixed in this population, we evidenced that other genes contributed, alone or within organised biological pathways, to explain the variability in carcass and meat quality traits. The information acquired might be incorporated into selective breeding programmes.

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Introduction

Meat quality is a complex phenotype, which includes several sensory attributes, such as tenderness, juiciness and colour, which are major drivers of consumer acceptance and product pricing (Grunert *et al.*, 2014).

Several factors related to environmental conditions and animal genetic background control meat quality. Although most of the meat quality traits have been found to be heritable (Johnston *et al.*, 2003; Boukha *et al.*, 2011), difficulties and costs related to phenotype collection make improvement through traditional selection unfeasible, so interest has shifted to genomic applications. Genome-wide association studies (**GWAS**) have made it possible to identify the single-nucleotide polymorphisms (**SNPs**) associated with the genes influencing carcass and meat quality traits in different conventional beef cattle breeds, which has improved our

understanding of trait biology and provided a list of positional candidate genes for quantitative trait loci (e.g., Tizioto *et al.*, 2013; Song *et al.*, 2016; Xia *et al.*, 2016; Santiago *et al.*, 2017; Zhang *et al.*, 2019). In addition, functional analyses coupled with GWAS have shown that a set of interacting genes and pathways are co-associated with carcass and meat quality traits (e.g., Ramayo-Caldas *et al.*, 2016). However, genomic selection and GWAS rely on linkage disequilibrium (**LD**) between the markers and quantitative trait loci affecting a target trait, which differ in different cattle populations and breeds (Ramayo-Caldas *et al.*, 2016).

The Piemontese breed is the most important Italian beef breed accounting for 330 000 animals, including 153 000 cows (Veterinary Information System, 2017). The distinctiveness of this breed is the double-muscled conformation induced by a specific mutation of the myostatin gene located on *Bos taurus* autosome (**BTA**) 2, which is almost fixed in this population. Piemontese animals have large muscular masses and low fat deposition, a low skeleton weight, low feed intake and good feed conversion (Fiems, 2012). Piemontese cattle are currently selected mainly for production traits (growth rate and muscularity) and direct and maternal calving ease (Albera *et al.*, 2004a). However, the recent award of EU Protected Geographical Indication status to 'Vitelloni Piemontesi della coscia' has increased interest in improving the meat quality attributes of Piemontese cattle.

So far, candidate-gene studies in the Piemontese breed have focused on identifying putative markers affecting carcass and meat quality traits (Lisa *et al.*, 2013; Ribeca *et al.*, 2014), but a complete genome-wide investigation for the same traits has never been carried out. The aim of this study, therefore, was to perform GWAS and pathway-based analyses of: (i) carcass traits (age at slaughter (**AS**), carcass weight (**CW**), carcass daily gain (**CDG**), conformation score (**EUS**) and rib-eye muscle area (**REMA**)); and (ii) meat quality traits (pH, Warner-Bratzler shear force (**WBSF**), purge loss (**PL**), cooking loss (**CL**) and colour parameters (lightness, **L***; redness, **a***; yellowness, **b***; chroma, **C***; hue, **H***)) in Piemontese young bulls in order to increase knowledge of the genomic regions and biological pathways controlling variation in these traits.

Material and methods

Animals and meat sample collection

This study was part of the Qualipiem project and involved 1369 Piemontese young bulls. The animals were fattened on 115 commercial farms representative of the beef production systems in the Piedmont region (north-west Italy), and slaughtered at the same commercial abattoir from April 2015 to February 2017. The beef farming systems, feeding regimes, fattening conditions and slaughter performances of the young bulls are described in detail in Savoia *et al.* (2019a). Briefly, the production systems were represented by traditional (restricted feeding and either tie-stalls or loose-housing), modern breeders and fatteners and specialised fatteners (the last two were divided in those using or not using total mixed rations). The chemical composition of feeds was obtained using analytical information for purchased commercial feeds, whereas for farm-produced feedstuffs, it was obtained using the chemical analysis of each feed ingredient (Sauvant *et al.*, 2004). Ground corn represented the main feed for all systems, consisting in 30% to 40% of the concentrate mix or total mixed ration administered to fattening animals. Ear corn silage was an important component of total mixed ration systems (from 19% to 30%). Hay was distributed *ad libitum* and used as forage in the traditional and modern systems with no total mixed ration, while a mixture of hay and wheat straw was used in total mixed ration systems. On average, the composition of concentrates was: 13% CP, 6% crude fibre, 4% ether extract and 5% ashes.

After slaughter, hot CW and carcass conformation according to the EU linear grading system (Commission of the European Communities, 1982) were assessed. To obtain more detailed differentiation, the six main grades (S, E, U, R, O, P from best to worst) were further subdivided to include + and − subclasses then converted into numerical scores (EUS) ranging from 18 (S+ class) to 1 (P- class). Carcass daily gain was computed as the ratio between CW and AS. Twenty-four hours after slaughter, individual samples of the *Longissimus thoracis* muscle were collected from between the fifth and sixth thoracic vertebrae and vacuum-packaged. Samples were then immediately transferred to the laboratory and stored at 4°C in a chilling room for 6 days until the assessment of beef quality traits.

Analysis of meat quality traits

At day 7 after animal slaughter, PL was determined according to the following procedure: the steaks were weighed initially in the bag (packaged weight, *W*1), then after bag removal (unpackaged weight, *W*3), and the bag was rinsed, dried and weighed (bag weight, *W*2). Purge loss (%) was then calculated as $(W1-W2-W3)/(W1-W2)*100$. pH was measured using a portable Crison pH meter equipped with a glass electrode suitable for meat penetration and an automatic temperature compensator. Colour was measured on the freshly cut surface of the steak after 1 h of blooming at 4°C using a Minolta CR-331C colorimeter. CIELAB coordinates (CIE, 1976), L*, a* and b* were recorded, H* was calculated as $H^* = \tan^{-1}(b^*/a^*)$, and C* as $C^* = (a^{*2} + b^{*2})^{0.5}$. Measurements were taken at three random locations on the meat surface and averaged. The steak was then sealed in a polyethylene bag and cooked in a water bath pre-heated at 75°C to an internal temperature of 70°C to assess CL. Once the set temperature was reached, the steak was removed from the water bath and cooled for 30 min under tap water. The steak was then removed from the bag, blotted and reweighed (Honikel, 1998). Cooking loss was computed as the percentage weight difference between the raw and cooked samples relative to the weight of the raw meat samples. The same steak was also used for the WBSF test. Six cylindrical cores 1.27 cm in diameter were taken parallel

to the muscle fibres and sheared perpendicular to their orientation with a V-shaped Warner-Bratzler blade fitted to an Instron Universal Machine model 5543. The Warner-Bratzler shear force was measured as the maximum force (Newtons) required to shear the cylindrical core at a cross-head speed of 200 mm*min^{-1} (AMSA, 2015).

Genotype data

The 1369 Piemontese young bulls were genotyped using the 'GeneSeek Genomic Profiler Bovine LD' (GGP Bovine LD) array containing 30 111 SNPs. According to most GWAS studies, SNPs on the X chromosome were excluded from the analyses. In current GWAS arrays, the density of markers in these regions is markedly lower than that of autosomes; moreover, analysis pipelines that have been developed for the autosomes cannot be directly applicable to X chromosome analyses (Wise *et al.*, 2013).

Quality control of genotype data was carried out using in-house pipelines and marker loci with minor allele frequencies <5% and call rate <95% were filtered out. In addition, in GWAS it is generally assumed that departure from Hardy-Weinberg equilibrium depends on potential genotyping errors (Marees *et al.*, 2018). Therefore, only SNPs which showed no extreme deviation from Hardy-Weinberg equilibrium ($P > 0.001$, Bonferroni corrected) were included in the analyses. The final marker set included 23 173 SNPs distributed on 29 BTAs. The SNPs positions were based on the UMD3.1 assembly (ftp://ftp.cbcb.umd.edu/pub/data/assembly/Bos_taurus/Bos_taurus_UMD_3.1/). A total of 203 animals were excluded based on the following criteria: (i) lack of concordance between SNP-based and pedigree-based ancestry; (ii) the herd they belonged to provided <3 animals; and (iii) there were <3 animals in their slaughter batch. The final number of animals included in the analyses was 1166.

Genome-wide association analysis

Genome-wide association analyses were conducted with a single marker regression model in the GenABEL R package using the GRAMMAR-GC (genome-wide association using Mixed Model and Regression – Genomic Control) approach (Amin *et al.*, 2007). Firstly, an additive polygenic model with a genomic relationship matrix was fitted:

$$\mathbf{y} = \mathbf{X}\beta + \mathbf{a} + \mathbf{e} \qquad (1)$$

where **y** is the vector of observations for each trait; β is the vector of non-genetic fixed effects included in the model: (1) slaughter batch (106 levels) and (2) herd (98 levels); **X** is the incidence matrix that associates each observation to specific levels of the factors in β. The two random terms included in the model were animal and the residuals, which were assumed to be normally distributed as $\boldsymbol{a} \sim N(0, \mathbf{G}\sigma_g^2)$ and $\boldsymbol{e} \sim N(0, \mathbf{I}\sigma_e^2)$, where **G** is the genomic relationship matrix, **I** is an identity matrix and σ_g^2 and σ_e^2 are the additive genomic and residual variances, respectively. The **G** matrix was constructed in the GenABEL R package, where for a given pair of individuals *i* and *j*, the identity by state coefficient ($f_{i,j}$) is calculated as:

$$f_{i,j} = \frac{1}{N}\sum_k \frac{(x_{i,k} - p_k) \times (x_{j,k} - p_k)}{p_k \times (1 - p_k)} \qquad (2)$$

where N is the number of markers used, $x_{i,k}$ is the genotype of the ith individual at the kth SNP (coded as 0, ½ and 1), p_k is the frequency of the '+' allele and $k = 1, \ldots, N$.

In a second step of GRAMMAR-GC, the residuals obtained in (1) are regressed on the SNPs (single marker regression) to test for associations. Amin *et al.* (2007) showed the power of GRAMMAR-GC approaches respect to that of the 'gold standard' methods for some pedigree structures in their study. Finally, the GC approach corrects for the conservativeness of the GRAMMAR procedure and estimates the marker effects. A *P*-value threshold of 5×10^{-5} was adopted to determine significant associations. Manhattan plots were drawn using the R package qqman (Turner, 2014). The variance explained by each SNP (σ^2_{SNP}) was calculated as $2pqa^2$, where p is the frequency of one allele, $q = 1 - p$ is the frequency of the second allele, and a is the estimated additive genomic effect. The proportion of genomic variance explained by each SNP was calculated as $\sigma^2_{g_{SNP}} = \frac{\sigma^2_{SNP}}{\sigma^2_g} \times 100$. Model (1) was also used to estimate variance components and the genomic heritability of the traits based on the genomic relationship matrix. Heritability was estimated as $h^2 = \frac{\sigma^2_g}{\sigma^2_g + \sigma^2_e}$.

For target regions, the Haploview v.4.2 software package (Barrett *et al.*, 2005) was used to estimate and plot pairwise LD measures (D' and r^2) and to define LD blocks according to the Gabriel criteria (Gabriel *et al.*, 2002).

Pathway analysis

Pathway analysis was performed to capture the weaker but related single-variant signals that were missed by standard GWAS due to the stringent *P*-value threshold. It relies on the assumption that complex traits might be controlled by changes in biological pathways or cellular functions in which many highly coordinated genes might play a modest role.

Therefore, we used a nominal *P*-value < 0.05 to select 'relevant' SNPs from the GWAS results. Then, using the R package BiomaRt (Durinck *et al.*, 2005), these SNPs were assigned to a gene if they were located within a gene or at a distance <15 kb from the coding region based on the Ensembl *Bos taurus* UMD3.1 assembly as the reference. For each trait, functional enrichment analyses were carried out on the list of significant genes using the Cytoscape plugin ClueGo (Bindea *et al.*, 2009) and the Kyoto Encyclopaedia of Genes and Genomes (**KEGG**) and the Gene Ontology (**GO**) databases. For each functional category, a right-sided hypergeometric test was used to reveal overrepresentation of significant gene sets. The Benjamini & Hochberg correction for multiple testing was used, and the cut-off for significant enrichment was set at a false discovery rate (**FDR**) <0.05.

The background SNPs/genes for the enrichment analyses represented all the SNPs/genes tested in the GWAS study.

Results

Descriptive statistics

Descriptive statistics for the carcass and meat quality traits are reported in Table 1. The average CW of the Piemontese young bulls sampled was 438.03 (±45.40) kg, while the average AS was 539 (±61.87) days, giving an average CDG of 0.82 (±0.11) kg/day. The average CW was relatively high with respect to AS due to the high carcass yield typical of this breed and the effect of myostatin mutation. Average EUS was 14.69, corresponding to an average evaluation approaching 'E+' in the EU linear grading system. The REMA was 92.2 (±14.21) cm^2 on average.

Considering meat quality traits, meat pH was 5.55 (±0.06). The two parameters related to meat water holding capacity, that is, DL and CL, were 4.51 (±1.19) % and 16.76 (±3.43) %, respectively. The Warner-Bratzler shear force value averaged 41.19 N, and it was characterised by high variability. Meat colour parameters averaged 39.91 (L*), 28.59 (a*), 9.64 (b*), 30.19 (C*) and 18.48 (H*). Carcass traits were strongly affected by production system (Savoia *et al.*, 2019a) with traditional management conditions having lower production efficiency. However, production system exerted only a very small effect on meat quality, limited to colour traits suggesting that future improvement should look, in particular, to genetics.

Table 1 *Descriptive statistics for carcass and meat quality traits in Piemontese cattle (n = 1166)*

Trait[1]	Mean	SD	h^2_g	#SNPs
Carcass traits				
AS (day)	539	61.87	0.151	3
CW (kg)	438.03	45.40	0.443	20
CDG (kg/day)	0.82	0.11	0.517	23
EUS	14.69	1.54	0.214	2
REMA, cm^2	92.22	14.21	0.486	0
Meat quality traits				
pH	5.55	0.06	0.072	1
Water holding capacity				
PL (%)	4.51	1.19	0.139	1
CL (%)	16.76	3.43	0.081	2
Colour parameters				
L*	39.91	3.52	0.386	1
a*	28.59	1.80	0.197	2
b*	9.64	1.70	0.247	2
C*	30.19	2.21	0.212	1
H*	18.48	2.14	0.266	2
WBSF,N	41.19	11.56	0.221	0

h^2_g: genomic heritability; #SNPs: number of significant (5×10^{-5}) single nucleotide polymorphisms for each trait.
[1] AS = age at slaughtering; CW = carcass weight; CDG = carcass daily gain, carcass; EUS = carcass conformation according to the EU linear grading system (Commission of the European Communities, 1982). The six main grades (S, E, U, R, O, P from best to worst) were further subdivided into + or – subclasses and then converted into numerical scores (EUS) ranging from 18 (S+ class) to 1 (P– class); REMA = rib-eye muscle area; PL = purge loss; CL = cooking loss; L* = lightness; a* = redness; b* = yellowness; C* = chroma; H* = hue; WBSF = Warner-Bratzler shear force.

Genome-wide association analysis

Genomic heritabilities for the carcass and meat quality traits are reported in Table 1. The highest genomic heritabilities (> 0.38) were detected for some carcass traits (CDG, REMA and CW) and for meat L*. The lowest heritabilities were found for meat pH and CL (<0.10), while the values of the remaining traits were intermediate (0.14 to 0.27).

The results of the GWAS analysis are summarised in Table 2 and Supplementary Table S1. We detected a total of 37 significant SNPs for 12 traits. No SNP passed the significance threshold for REMA and WBSF. The *P*-values ranged from 4.83×10^{-5} to 2.44×10^{-11}. The traits with the highest numbers of significant SNPs were CDG (23) and CW (20).

Regarding carcass traits, we detected significant associations on BTA3 (at ~31.52 Mb) for CDG; on BTA6 for AS, CW, CDG (at ~38.46 to 40.56 Mb and 91.91 Mb) and EUS (at ~71.15 Mb); on BTA11 (at ~94.69 Mb) for EUS; and on BTA19 (at ~6.90 Mb) for CW.

For meat colour parameters, we detected signals on BTA4 (at ~112.51 Mb), BTA23 (at ~3.91 and ~7.25 Mb), BTA24 (at ~19.87 Mb) and BTA25 (at ~11.96 Mb). This last signal, in particular, corresponded to the marker BovineHD2500003345, which was associated with four out of the five colour parameters assessed (i.e., a*, b*, c*, H*). Regarding the traits related to water holding capacity, one SNP located on BTA9 (at ~48.33 Mb) was significant for PL, and two SNPs located on BTA6 (at ~29.23 Mb) and on BTA10 (at ~14.57 Mb) were significant for CL. Finally, one SNP located on BTA8 (at ~28.46 Mb) was associated with meat pH.

The highest signals were associated with CDG and corresponded to the markers ARS-BFGL-NGS-45457 ($P = 2.44 \times 10^{-11}$) and Hapmap26308-BTC-057761 ($P = 6.37 \times 10^{-9}$), which mapped onto BTA6 at ~38.72 and 38.52 Mb, respectively. These SNPs explained 9.61% (ARS-BFGL-NGS-45457) and 7.31% (Hapmap26308-BTC-057761) of the additive genetic variance for CDG, and were in strong LD (Supplementary Table S1 and Supplementary Figure S1). They were included in a window of 23 SNPs located on BTA6 in the range ~38.46 to 40.56 Mb, which showed significant associations for CDG, CW and AS (Table 2; Figure 1). Several genes mapped to this region. In particular, 14 SNPs located at ~38.57 to 38.94 Mb mapped within four genes: Leucine Aminopeptidase 3 (***LAP3***) (seven SNPs), Family with Sequence Similarity 184 Member B (***FAM184B***) (two SNPs), Non-SMC Condensin I Complex Subunit G (***NCAPG***) (two SNPs) and Ligand-Dependent Nuclear Receptor Corepressor-Like (***LCORL***) (three SNPs) (Table 3). The marker BovineHD0600010666 was located within 2kb 5'-UTR of *LAP3*, and the markers Hapmap26308-BTC-057761 and BovineHD0600010673 corresponded to intron variants of *LAP3*. In addition, four SNPs, that is, MS-rs110839532, MS-rs43702361, MS-rs109241256 and MS-rs41255599, were located within the 3'-UTR of this gene. The markers

Table 2 *Results of genome-wide association analysis for carcass and meat quality traits in Piemontese beef cattle (n = 1166)*

BTA	#SNPs	*P*-value (range)	Top SNP	Top SNP location (bp)	Top SNP MAF	Trait[1]
3	1	3.75×10^{-5}	ARS-BFGL-NGS-76281	31524593	0.39	CDG
4	1	3.34×10^{-5}	BovineHD0400032408	112507562	0.46	H*
6a	1	4.04×10^{-5}	BovineHD0600008146	29225507		CL
6b	23	2.44×10^{-11}, 4.55×10^{-5}	ARS-BFGL-NGS-45457	38715250	0.43	**CDG**, CW, AS
6c	1	9.69×10^{-6}	BTA-76623-no-rs	71154473	0.12	EUS
6d	1	1.78×10^{-5}	Hapmap49816-BTA-98191	91906227	0.18	AS
8	1	2.02×10^{-5}	ARS-BFGL-NGS-114722	28464160	0.19	pH
9	1	1.54×10^{-5}	BovineHD0900013319	48330996	0.43	PL
10	1	4.15×10^{-5}	ARS-BFGL-NGS-70946	14574453	0.42	CL
11	1	4.83×10^{-5}	ARS-BFGL-NGS-116123	94686959	0.32	EUS
19	1	3.07×10^{-5}	ARS-BFGL-NGS-4893	6895198	0.43	CW
23	1	4.47×10^{-5}	BovineHD2300000877	3907142	0.36	a*
23	1	3.56×10^{-5}	BovineHD2300001826	7245409	0.37	L*
24	1	4.58×10^{-5}	BovineHD2400005258	19872257	0.38	b*
25	1	7.79×10^{-6}, 3.53×10^{-5}	BovineHD2500003345	11960157	0.27	**a***,b*,c*,H*

BTA = *Bos taurus* autosome chromosome; #SNPs = number of the single nucleotide polymorphisms significantly associated to the trait; *P*-value (range) = The *P*-value of the highest significant SNP adjusted for genomic control and the range of the *P*-values when multiple SNPs were significantly associated to one trait; Top SNP location (bp) = position of the highest significant SNP on the chromosome in base pairs on UMD3.1 (http://www.ensembl.org/index.html); Top SNP MAF = minor allele frequency of the top SNP.
The trait with the highest *P*-value in each genomic region is bolded.
[1] See Table 1 for the traits abbreviation description.

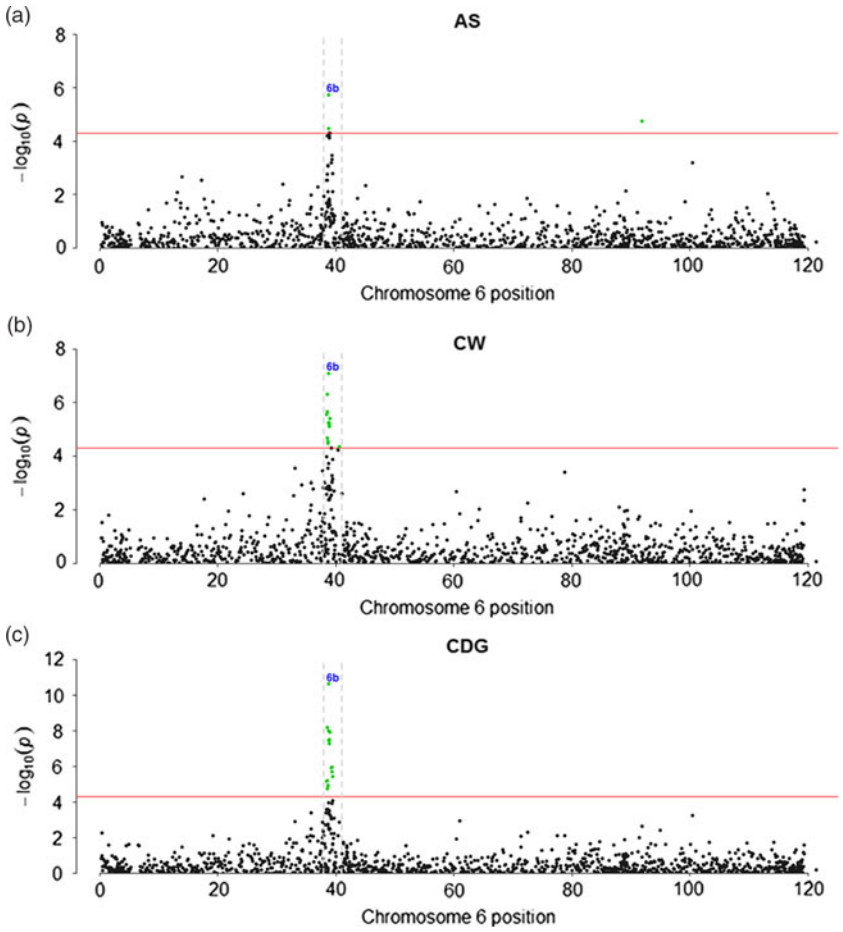

Figure 1 (Colour online) Manhattan plots for the genome-wide association results of (a) age at slaughtering, (b) carcass weight and (c) average daily gain in carcass on *Bos taurus* autosome 6 (**BTA6**) in Piemontese beef cattle. AS = age at slaughtering; CW = carcass weight; CDG = carcass daily gain. The red horizontal lines indicate a –log10 (*P*-value) of 4.30 (corresponding to *P*-value = 5×10^{-5}). The region 6b of BTA6 is highlighted.

Table 3 *Significant SNPs mapping in the region 38.57 to 38.94 Mb on Bos taurus autosome (BTA) 6 in Piemontese beef cattle*

SNP	CHR	BP	*P*-value	MAF	Gene	Variant effect[1]
BovineHD0600010666	6	38574125	1.73×10^{-5}	0.48	*LAP3*	nearGene-5'[2]
Hapmap26308-BTC-057761	6	38576012	4.81×10^{-7}	0.38	*LAP3*	intron
BovineHD0600010673	6	38590515	3.25×10^{-5}	0.48	*LAP3*	intron
MS-rs110839532	6	38599667	3.25×10^{-5}	0.48	*LAP3*	3'UTR
MS-rs43702361	6	38599672	1.32×10^{-5}	0.48	*LAP3*	3'UTR
MS-rs109241256	6	38599864	2.79×10^{-5}	0.48	*LAP3*	3'UTR
MS-rs41255599	6	38599993	3.25×10^{-5}	0.48	*LAP3*	3'UTR
BovineHD0600010685	6	38616248	3.25×10^{-5}	0.48	*FAM184B*	intron
ARS-BFGL-NGS-45457	6	38715250	1.86×10^{-6}	0.43	*FAM184B*	intron
MS-rs109570900	6	38777311	3.35×10^{-5}	0.46	*NCAPG*	missense
MS-rs110251642	6	38808241	5.98×10^{-6}	0.48	*NCAPG*	missense
BovineHD0600010755	6	38866381	5.33×10^{-8}	0.48	*LCORL*	intron
Hapmap31285-BTC041097	6	38869785	5.98×10^{-6}	0.48	*LCORL*	intron
Hapmap33628-BTC041023	6	38939012	1.23×10^{-8}	0.46	*LCORL*	intron

SNP = single nucleotide polymorphism; CHR = chromosome; BP = SNP location in bp; MAF = minor allele frequency; Gene = gene in which SNP is located; *LAP3* = Leucine Aminopeptidase 3; *FAM184B* = Family with Sequence Similarity 184 Member B; *NCAPG* = Non-SMC Condensin I Complex Subunit G; *LCORL* = Ligand Dependent Nuclear Receptor Corepressor Like.
[1] Variant effect: SNP effect on the gene.
[2] ~0.5 kb upstream of *LAP3*.

BovineHD0600010685 and ARS-BFGL-NGS-45457 were intron variants of *FAM184B*.The markers MS-rs109570900 and MS-rs110251642 corresponded to missense mutations in *NCAPG*. Finally, three SNPs, that is, BovineHD0600010755, Hapmap31285-BTC-041097 and Hapmap33628-BTC-041023, were intron variants of *LCORL* (Table 3). Pairwise measures of LD in this region are reported and visualised in Supplementary Figure S1.

Pathway analyses

In total, 14 265 SNPs (out of the 23 173 SNPs in the chip) were located in annotated genes or within a 15 kb window surrounding the coding region. On the basis of the *Bos taurus* UMD3.1, 9713 annotated genes were used as background for the pathway analysis.

For each trait, ~700 significant SNPs ($P<0.05$) were on average assigned to ~600 genes, which were mined using Cluego (Bindea *et al.*, 2009) to identify the biological pathways and cellular functions involved in controlling carcass and meat quality traits.

Significantly enriched GO terms and KEGG pathways (FDR<0.05) were found for four meat quality parameters, that is, a*, b*, C* and CL, and for two carcass traits, that is, CW and EUS (Figure 2 and Supplementary Table S2). In particular, for the meat colour parameters we observed a high association between the pathways and GO terms related to transmembrane transport activity. For instance, calcium channel activity was commonly enriched among a* (FDR = 0.0137), b* (FDR = 0.0214) and C* (FDR = 0.0348); inorganic cation transmembrane transport was overrepresented for both a* (FDR = 0.0470) and C* (FDR = 0.0438); oxygen transport (FDR = 0.0475) and inorganic ion transmembrane transport (FDR = 0.0458) were associated to C*. In addition, a set of genes pertaining to the dopaminergic synapse were specifically enriched for a* (FDR = 0.0421); ribonucleoside bisphosphate biosynthetic process, and purine nucleoside bisphosphate biosynthetic process were highly associated with b* (FDR = 0.0195); circadian entrainment (FDR = 0.0434) and long-term depression (FDR = 0.0477) pathways were enriched for C*. Finally, the regulation of synapse assembly was overrepresented for CL (FDR = 0.0274). Regarding carcass traits, a set of 5 genes involved in protein localisation to synapse were enriched for CW (FDR = 0.0292), while a set of 31 genes involved in response to organic cyclic compound were enriched for EUS (FDR = 0.0292).

Discussion

This is the first study combining GWAS and biological pathway analysis for economically important traits related to carcass and meat quality traits in the Piemontese breed.

The magnitude of genomic heritability for carcass traits was higher than previous estimates based on pedigree analysis for the same traits in a field survey on this breed (Boukha *et al.*, 2011). The genomic estimates in this study were also generally higher than pedigree-based estimates obtained from the same dataset, with the exception of AS, pH, CL and WBSF (Savoia *et al.*, 2019b). Overall, these results provide further support for the existence of exploitable genetic variation in the carcass and meat quality traits of young bulls.

Genome-wide association study

For the GWAS study, we used the GRAMMAR-GC approach which is a powerful and fast method for GWAS analyses in case of related individuals. This approach exploits genomic marker data for correction of the distribution of the test statistics and for inferring the relationships among individuals (Amin *et al.*, 2007).

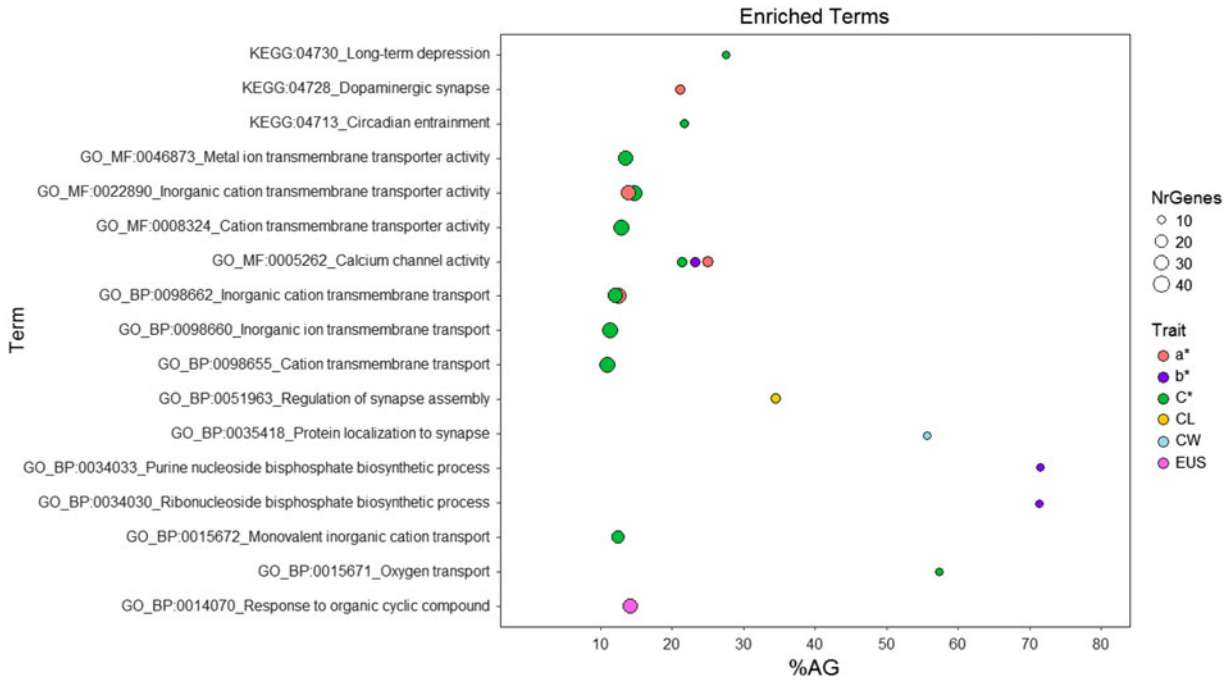

Figure 2 (Colour online) Significantly enriched gene ontologies (**GO**) terms and Kyoto Encyclopaedia of Genes and Genomes (**KEGG**) pathways for carcass and meat quality traits in Piemontese beef cattle. Only the significant terms are displayed (FDR<0.05). %AG=percentage of genes associated with the significant pathways with respect to the total number of genes in the pathway; CW=carcass weight; CL=cooking loss; EUS=carcass conformation according to the EU linear grading system (Commission of the European Communities, 1982). The six main grades (S, E, U, R, O, P from best to worst) were further subdivided into + or – subclasses and then converted into numerical scores (**EUS**) ranging from 18 (S+ class) to 1 (P- class); a*: redness; b*: yellowness; C*: chroma. The size of the circles corresponds to the number of significant genes for each term, and the colour of the circle corresponds to the trait of interest.

The results obtained with GWAS analyses showed that aside from the role of myostatin gene mutation, which is fixed in the Piemontese population, other markers/genes contribute to explaining the variability in carcass traits in this highly specialised beef breed.

We detected 14 SNPs associated with CW, CDG and AS on BTA6 (Table 3), which were mapped within four genes, that is, *LAP3*, *FAM184B, NCAPG* and *LCORL*. Leucine Aminopeptidase 3 is involved in the control of oxytocin hydrolysis and has been associated with milk protein and fat content in dairy cows (Zheng *et al.*, 2011). Little information is available for *FAM184B*, but it has been suggested that it is associated with feed intake and weight gain in crossbred cattle (Snelling *et al.*, 2011). Polymorphism in *NCAPG* exhibited significant associations with CW, estimated carcass yield and *Longissimus* muscle area in Japanese Black cattle (Hoshiba *et al.*, 2013). Ligand Dependent Nuclear Receptor Corepressor Like has been shown to control stature in cattle in association with *NCAPG* (Pryce *et al.*, 2011). Significant SNPs for bone weight have been located in or near *LAP3*, *LCORL*, *FAM184B* and *NCAPG* in Simmental cattle (Xia *et al.*, 2017). In particular, the most significant SNP found by the latter authors (Hapmap26308-BTC-057761) was also found to have the second-strongest association with CDG (and with CW, although to a less extent) in the present study, and with CW in Japanese Black cattle (Nishimura *et al.*, 2012). Moreover, these genes have been considered potential positional and functional candidate genes for direct calving ease in Piemontese cattle (Bongiorni *et al.*, 2012). The same SNP, that is, Hapmap26308-BTC-057761 (intron variant of *LAP3*) was associated to CW and CDG in our study, and to direct calving ease in the study of Bongiorni *et al.* (2012) study. In addition, the markers MS-rs109570900 and MS-rs110251642 (missense mutations in *NCAPG*) were associated with AS, CW and CDG (present study) and direct calving ease (Bongiorni *et al.*, 2012). Calving performance has two components: the ability of the dam to give birth easily (maternal effect) and the ability of the calf to be born easily (direct or foetal effect). Direct effects are primarily connected to the size of the calf, which might explain the associations found in the present study with muscularity and growth parameters. This interpretation is confirmed by a study carried out by Albera *et al.* (2004b), who estimated consistent positive genetic correlations between daily gain by young bulls and direct calving difficulties in first and later parity Piemontese cows.

The marker BovineHD2500003345 (located at ~11.96 Mb on BTA25), which was associated with meat colour parameters, was an intron variant of Shisa family member 9, which maps in quantitative trait loci associated with traits of economic importance in bovines (http://www.animalgenome.org/cgi-bin/QTLdb/BT/index), such as BW, but also with clinical mastitis, (maternal) dystocia and milk protein percentage and yield. Moreover, the marker BovineHD2300001826, which was associated with a*, corresponded to a synonymous mutation in the coding sequence of bromodomain containing 2. This gene is a nuclear serine/threonine kinase involved in transcriptional regulation and has been suggested as a candidate gene for meat quality parameters, including meat colour (a*), in pigs (Lee *et al.*, 2018).

Variation in calpain 1 (***CAPN1***) and calpastatin (***CAST***), which are involved in an important proteolytic system, has been associated with beef tenderness in different breeds (Tizioto *et al.*, 2013; Ramayo-Caldas *et al.*, 2016). Although markers located within these genes were included in the chip (UA-IFASA-1370, intron variant of *CAPN1*; BovineHD0700028726, BovineHD0700028737 and ARS-USMARC-116, intron variants of *CAST*; BovineHD0700028758, variant within 2kb upstream of *CAST*; ARS-USMARC-670, synonymous mutation in the coding sequence of *CAST*), no significant association was detected with WBSF in this study. This is in line with previous results from a candidate gene study, which reported no significant associations for markers located in *CAPN1* and *CAST* with SF in Piemontese cattle (Ribeca *et al.*, 2013). A possible explanation might be that in double-muscled breeds myostatin mutation plays a major role in the regulation of meat tenderness, while other genes make only a marginal contribution. Indeed, consistent with its function as a regulator of muscle cell growth and differentiation, myostatin has been identified among the top transcription factors for meat quality traits, including tenderness, in French beef cattle breeds (Ramayo-Caldas *et al.*, 2016). However, it is worth mentioning that other authors did not detect any significant SNP on *CAST* or *CAPN1* associated with WBSF in Simmental cattle (Xia *et al.*, 2016).

Pathway analysis

Meat quality is dependent on biochemical and biophysical changes occurring in the muscle *postmortem*. The results of our pathway analysis show that calcium, ion and cation transport pathways as well as oxygen transport were enriched for meat colour parameters. Calcium is essential for muscle contraction by acting as a catalyst of enzymatic proteolytic activity, and metabolic pathways related to calcium transport have been associated with meat quality, in particular tenderness (Ramayo-Caldas *et al.*, 2016). Moreover, seasonal variation in L* values (more pale muscle in the summer) has been attributed to changes in the Ca^{2+} release channels (Küchenmeister *et al.*, 2000). Several potassium channels and solute carriers were also included in the enriched pathways. Indeed, K^+ is necessary for muscle contraction and nerve impulses, and together with sodium it contributes to maintaining fluid balance in the cells (Knochel and Schlein, 1972). Moreover, K^+ content has been reported to influence meat quality traits, that is, tenderness (Tizioto *et al.*, 2014). Our results seem to suggest that K^+ transport might also play a role in controlling beef colour. The enrichment of oxygen transport observed for C* might be related to *postmortem* aging. Increased aging has been reported to improve blooming by decreasing the competition between mitochondria and myoglobin for oxygen, and by improving myoglobin oxygenation (MacDougall, 1982). Aging can also influence cellular mechanisms (such as oxygen scavenging and reducing enzymes), which are critical to meat colour stability (Nair *et al.*, 2018). It is worth mentioning that a greater heme/iron content was reported in meat from the Piemontese breed compared with other European beef cattle breeds (Chambaz *et al.*, 2001). In addition, variations in the genes involved in heme metabolism have been found between the Piemontese and Marchigiana breeds (Sorbolini *et al.*, 2015). Finally, a close link between purine metabolism in skeletal muscle and its physical and chemical properties, including colour, has recently been found (Zheng *et al.*, 2018), which might explain the overrepresentation of the purine nucleoside bisphosphate biosynthetic process for b*.

Several genes involved in gamma-aminobutyric acid (**GABA**)ergic signalling were included in the response to the organic cyclic compound biological process, which was enriched for EUS; these included some GABA receptors, namely *GABRB1*, *GABRB3* and *GABRG2*, as well as a down-stream G-protein, G protein subunit gamma 2, and a protein kinase C, protein kinase C Gamma. Gamma-aminobutyric acid is the main inhibitory neurotransmitter in the mammalian central nervous system. It is synthesised from glutamate by the enzyme glutamic acid decarboxylase and was reported to play a role in controlling feeding behaviour in ruminant animals (Seoane *et al.*, 1984). The GABAergic synapse pathway has also recently been associated with live weight in Simmental cattle (Fan *et al.*, 2015).

More difficult to interpret, however, is the enrichment of the regulation of synapse-related pathways for CL and CW, although it is notable that the genes associated to CL included Wnt family member 5A and Wnt family member 7A, which have been associated with meat quality, muscle fibre types and *postmortem* energy metabolism in pigs (Men *et al.*, 2017).

In summary, the results of this study have allowed us to identify genomic regions and biological pathways controlling carcass and meat quality traits in the Piemontese breed. In particular, we showed that while myostatin plays the major role, other genes also contribute to explaining variability in carcass and growth characteristics. Of particular interest is the finding that the pathways related to transporter activity (oxygen, calcium, ion and cation) are associated with meat colour parameters. The results obtained provide useful knowledge that may be exploited in developing selection programmes aimed at improving meat production and quality. In particular, the information acquired with the present study might be included into prediction models as biological prior to increase the knowledge about the genetic control of meat production and quality traits and improve the accuracy of genomic prediction.

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Declaration of interest

Authors declare no conflict of interest.

Ethics statement

Animal Care and Use Committee approval was not obtained for this study because the data were obtained from an existing database; the analysed records were collected after slaughtering of animals in a commercial abattoir (Operti, Centallo (CN), Italy) from April 2015 to February 2017. The authors did not have direct control over the care of the animals included in this study.

Software and data repository resources

None of the data were deposited in an official repository.

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S1751731119001812.

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