Final Report

Genom-wide association study (GWAS) for the improvement of beef quality and palatability traits in Hungarian Simmental cattle- Project 111643

The main aim of modern livestock agriculture is to satisfy growing demands of consumers worldwide for high-quality, safe and delicious animal products. This trend is evident for the beef industry as well. Primary goal is to produce a quality beef product that consumers are willing to purchase; a tender, juicy, and flavourful product of good value. Among the palatability traits that govern eating quality of beef, tenderness is widely regarded as the most important factor influencing customers' satisfaction and subsequent repurchasing of beef. Intramuscular fat content (IMF) or marbling has a positive influence on tenderness, along with other palatability traits such as juiciness and flavour. Nowadays the main objective of numerous studies is improvement of IMF in beef. Consequently, analysis of marbling plays an important role in the evaluation of meat quality. Marbling score is a component of several beef quality grading systems in the world including the United States, Japan and Australia. It refers to visible fat found between muscle fibre bundles within the longissimus dorsi (LD) muscle. Marbling score is assessed subjectively (visual appraisal) by a grader during the process of assigning beef quality grade. Chemical analysis is another conventional method for meat marbling evaluation, and it has been widely used as a standard method for the accurate determination of IMF content. Intramuscular fat simply quantifies the amount (percentage) of fat within the muscle, but marbling score considers amount, distribution and texture of fat, too. In Europe, either IMF level or other meat quality traits have not yet been introduced in the EUROP-based grading system.

With such a variety of factors influencing tenderness, improvement and prediction of this trait is extremely difficult. Moreover, the rapid and non destructive measurement of tenderness is not actually available. The main methods currently employed to predict beef tenderness are consumer taste panel assessment and the Warner–Bratzler shear force (WBSF) method. Sensory panel analysis is a subjective evaluation method. This method of taste sensitivity is time-consuming and has unfortunately poor repeatability. The WBSF method can be used to objectively determine mechanical beef tenderness. Despite of being objective and accurate it is difficult to apply it in meat industry. Therefore, it was necessary to develop a non-destructive, efficient and rapid inspection method for beef quality. Advances in non invasive digital imaging techniques allow genotype-based beef selection. The muscle density measured by CT is indicative for the intramuscular fat, which is a major indicator of taste of meat and possibly for tenderness.

As previous mentioned, the ability to predict and to measure eating quality traits including meat tenderness and marbling is one of the major research goals in beef production. At present, there is considerable interest in the application of molecular technologies for specific DNA markers associated with various QTL (Quantitative Trait Loci) to promote more efficient and relatively easy selection and breeding programme in farm animals. In the last few years advances in molecular genetics have enabled the application of Genomic Selection in achieving different breeding objectives. Several QTLs for performance and beef production have been identified, and a number of potential candidate genes have been selected for analysis based on a known relationship with physiological or biochemical processes and production traits.

In our research we used genom-wide association study (GWAS) for the improvement of beef quality traits and some essential breeding values which signify level of breeding potential of animals for specific traits. SNP typing was performed on high-resolution SNP chips developed for cattle (Illumina Bovine HD Chip). Tissue composition and intramuscular fat content of muscle samples were determined by digital cross-section imaging techniques and analytical methods. Beef quality traits were analyzed by conventional laboratory methods. Correlations between the obtained SNP genotypes and laboratory and CT results were highlighted by statistical analysis. Subject of present research and selection of animals included in this study were elaborated with the assistance of specialists from Association of Hungarian Simmental Cattle Breeders (AHSCB). Production data, breeding parameters as well as fattening performance data of all animals included in the project were collected from the database of the AHSCB.

In the first year of the project 68 different sperm samples from sires and grandsires were collected from the Gene Bank of the Association of Hungarian Simmental Cattle Breeders. Besides, 52 offspring of selected bulls were fattened at experimental farms of Simmental breeders and slaughtered at a commercial slaughterhous. In the second and third year of the experiment number of fattened and slaughtered animals was 48 and 72, respectively. So total number of genotyped animals included in this study was higher as scheduled (240 vs 200).

Animals were fed and kept in identical conditions and were slaughtered at approximately similar live weight (530.6±44.7 kg) under commercial conditions using Hungarian standard procedure. Slaughter weight, hot and cold carcass weight and EUROP classification scores were recorded prior to slaughter and on the slaughter line. After 24 hours chilling, meat samples have been collected from the right half carcass m. longissimus dorsi (LD) cut at the 12^{th} rib, m. semitendinousus (SM) and m. psoas major (PM) for cross sectional imaging and laboratory analysis. Muscle samples were wrapped in foil and stored at 4^{o}C degrees until analysis. Blood samples have been collected during exsanguinations and were stored at -20^{o}C until DNA extraction.

Considering that intramuscular fat content (IMF) is an economically important factor in many beef carcass classification systems, marbling traits of LD muscle have been evaluated with different methods:, by image analysis of X-ray computed tomography (CT) scans, by USDA marbling score and by conventional analytical method.

CT examinations were carried out with the usage of a 16-slice CT system (Siemens Somatom Sensation Cardiac 16). Usually, meat samples were scanned at voltage of 120 kV, but in this case various user-selectable tube voltages were used (e.g. 80 and 140 kV). According to our opinion, the fusion of scans made at different kV settings resulted in a higher accuracy concerning separation of different tissues. CT value at LD muscle area of each mixed scan (80±140 kV) was obtained. Volumetric connective tissue content has been determined with the usage of MANGO (3.8, 2016) software. With simple thresholding, pixels between −200 and +19 CT value were included in the evaluation of IMF.

The MRI examinations were performed by 3 T Biograph PET/MR scanner. This system has a twice higher magnetic field than most commercially available scanners. Because of the highest possible resolution and signal to noise ratio, optimum image quality scans have been made. The measurement of T2 relaxation time was evaluated by developing some different protocols in order to assess intramuscular fat and connective tissue of beef. Results showed

positive association (r=0.64 n=16) between CT data (120 kV) and intramuscular fat content, whereas at CT scans, coefficient of correlation r= 0.52 was detected between hydroxyproline content and connective tissue proportion.

After the execution of cross sectional imaging techniques from the longissimus dorsi muscle, meat samples were submitted to laboratory examination, i.e. determination of IMF (Soxhlet-method), WBSF-measurement (Ruiz et al.), connective tissue determination (Reddy and Enwemeka).

Intramuscular connective tissue (IMCT) is a major factor affecting meat tenderness. The main component of IMCT is collagen and generally it has been called “background” toughness of meat. CT-number of collagen is not well documented in the literature, though a few human CT studies determine the collagen density to be higher than both in muscle and fat tissue.The ability to differentiate soft tissue structures within muscle is limited, because these mainly contain small atomic numbers and showed similar X-ray attenuation values. Based on our hypothesis this material differentiation is with the usage of dual energy more feasible.

Chemical fat content of LD varied between 0.5 and 7.9%, whereas CT fat content varied between 0.1 and 8%. IMF in the 12^{th} rib and in LD on CT-scans closely correlated with chemical fat content (r=0.69 and 0.80 respectively).

Hydroxyproline measurement is the most common method used to determine connective tissue (collagen) content of meat. Hydroxyproline content was determined by acidhydrolysis and total collagen was calculated as hydroxyproline ×7.25 and expressed as gram of collagen per 100g of muscle. Mean collagen content of bulls was 0.5%, whereas observed CT connective tissue content was 0.4%. A weak relationship was found between the IMF and collagen content as well as CT connective tissue proportion (r= - 0.3, r=- 0.32).

Table 1. and Table 2. summarise the carcass and longissimus muscle traits according to EUROP conformation and fat classes. The average hot carcass weight was 294 kg and it was a significant difference between conformation and fat grades. It seems that carcass weight increased with conformation class and fat grade, too. Mean values of EUROP fat grade in conformation classes varied from 4 (2-) to 6.1 (2+), whereas mean values of EUROP conformation grade in fat classes varied from 4.2 (O0) to 7.4 (R0). Higher fat grade paired with significantly more favourable conformation grade. The average live weight growth rate and net carcass gain of bulls were 955 g/d and 519 g/d, respectively. There were no significant differences between EUROP classes for these traits, but the growth rate tended to be higher in better EUROP conformation and higher fat grades.

The marbling traits of longissimus muscle were similar in different EUROP conformation classes. However fat grades differed significantly for the examined marbling traits, except for USDA marbling score.The average chemical fat content of LD varied between 2.2 and 3.1% in conformation classes, whereas from 2.3 to 5.0% in fat classes. Similar to chemical fat content, CT fat content was the highest in O conformation class and in 1. fat class. In line with higher CT fat content in longissimus muscle Hounsfield unit measured average CT value and CT muscle percentage decreased.

Marbling is an economically important factor in many beef carcass classification systems such as USDA. The highest frequency of USDA marbling score was "small" (55.2%), followed by "slight" (25.5%), "modest" (17.2%) and "moderate" (2.1%). The highest USDA marbling scores were given for bulls belonging to “O” and “P” conformation classes and to “3” fat class. Higher intramuscular fat level was found in lower conformation classes. The highest intramuscular chemical fat level and CT fat percentage of longissimus muscle was obtained by bulls classified as EUROP fat grade 1, followed by fat grade 3 and the lowest intramuscular fat level was measured in carcasses with EUROP fat grade 2.

Table 1. Carcass and longissimus muscle traits among EUROP conformation class

Traits	EUROP CONFORMATION GRADE								
	P		O		R		Overall mean		P-value
CARCASS	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Hot carcass weight, kg	220.7	49.7	268.6	52.6	360.3	93.2	293.7	84.4	***
EUROP fat grade(1-15)	4.0	1.8	5.1	1.0	6.1	1.4	5.3	1.4	NS
Net carcass gain, g/d	479.2	102.8	487.7	152.2	583.1	100.6	519.0	136.8	NS
Live weight gain, g/d	938.7	227.2	913.2	281.1	1026.1	207.1	955	252	NS
LONGISSMUS MUSCLE									
Marbling score, USDA*	2.0	0.6	2.0	0.8	1.8	0.7	2.0	0.7	NS
Intramuscular fat, %	3.0	1.9	3.1	2.1	2.2	1.7	2.8	1.9	NS
Average CT value, HU	67.0	3.7	64.3	5.3	63.9	4.0	64.5	4.7	NS
CT fat content, %	2.3	1.0	2.4	1.6	1.7	1.4	2.1	1.2	NS
CT muscle content, %	97.2	1.5	97.3	2.4	98.0	1.7	97.5	2.1	NS
CT fat area, mm^2	1281.9	523.4	1558.8	351.8	903.3	201.9	1331.2	358.8	NS

*1:slight, 2: small 3: modest

Table 2. Carcass and longissimus muscle traits among EUROP fat class

Traits	EUROP FAT GRADE						
	1		2		3		P-value
CARCASS	Mean	SD	Mean	SD	Mean	SD	
Hot carcass weight, kg	195.4	26.9	291.0	60.3	412.2	137.2	***
EUROP muscle grade (1-15)	4.2	2.5	5.6	1.4	7.4	0.6	**
Net carcass gain, g/d	442.5	209.3	518.9	122.8	596.7	144.2	NS
Live weight gain, g/d	882	422	947	215	1088	330	NS

Marbling score, USDA*	1.8	0.5	1.9	0.8	2.2	0.8	NS
Intramuscular fat, %	5.0	2.8	2.3	1.6	4.1	1.8	**
Average CT value, HU	61.2	8.5	65.4	3.8	61.2	4.3	*
CT fat content, %	3.8	3.3	1.1	1.0	2.9	2.4	**
CT muscle content, %	95.9	3.4	97.9	1.7	96.4	2.2	*
CT fat area, mm^2	2628.6	1264	890.6	182.9	1831.6	312.8	*

*1:slight, 2: small 3: modest

Table 3. presents the simple correlation matrix of examined traits. Carcass traits moderately correlated with each other, but did not relate to LD traits. USDA marbling traits loosely correlated with IMF values obtained by CT and Soxhlet method. The fat percentage and fat area in longissimus muscle on CT-scans closely correlated with chemical fat content (r=0.81 and 0.85 respectively). Negative correlation was detected between CT fat and CT muscle percentage, as well as average CT value and fat content. It is important to evaluate correlations between growth rate and marbling traits. It was found a negative relationship between live weight gain as well as net carcass gain and CT fat percentage as well as area. Opposite tendency can be seen between growth rates and CT muscle percentage.

Table 3. Simple correlation coefficients for examined traits

Traits	Hot carcass weight	EUROP muscle grade	EUROP fat grade	Net carcass gain	Live weight gain	Marbling score, USDA	Intramuscular fat	Mean CT value	CT fat content, %	CT muscle content	CT fat area
Hot carcass weight	1	0.59	0.65	0.34	-	-	-	-	-	-	-
EUROP muscle grade		1	0.52	0.36	-	-	-	-	-	-	-
EUROP fat grade			1	0.29	-	-	-	-	-	-	-
Net carcass gain				1	0.95	-	-0.33	-	-0.41	0.40	-0.48
Live weight gain					1	-		0.31	-0.42	0.39	-0.49
Marbling score, USDA						1	0.39	-	0.36	-0.43	0.32
Intramuscular fat							1	-0.58	0.81	-0.81	0.85
Mean CT value								1	-0.78	0.47	-0.74

CT fat content, %	1	-0.9	0.93
CT muscle content		1	-0.89
CT fat area			1

Although it was not primarily planned in this project, samples and slaughter data from 12 bull and 12 cow carcasses of Hungarian Simmental cattle were randomly collected at a commercial slaughterhouse and submitted to CT and laboratory examinations. Aim of analyses was comparison of CT and laboratory examination results of LD muscle from animals of different age and sex.

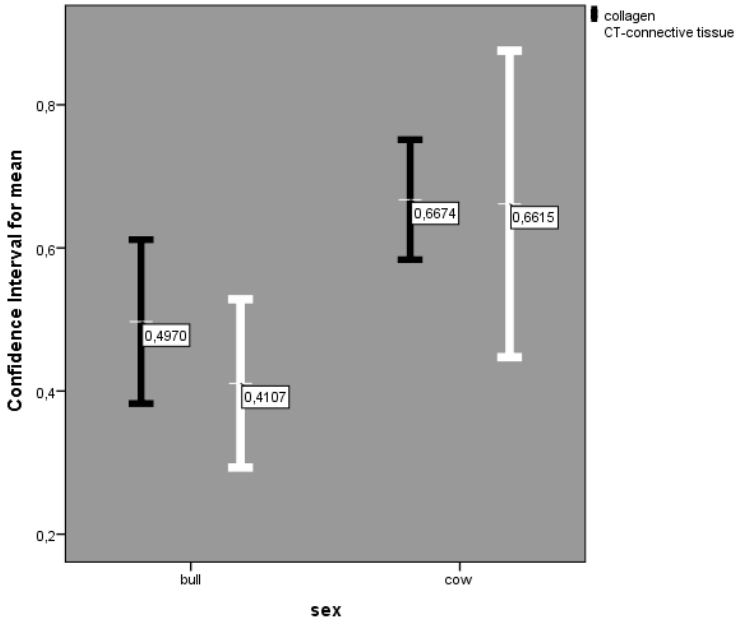
Table 4. Summary statistics of slaughter and carcass data

Variable		Hot carcass weight, kg	EU muscle score (P0:1; E+:15)	EU fat score (1-:1; 5+:15)	LT area, cm^2	IMF, %
Sex	bulls	294.7±31.2*	5.5±1.3*	5.9±0.4*	77.1±14.1	2.7±1.9
	cows	247.2±29.5**	3.6±1.7**	4.2±1.8**	71.1±11.7	2.8±2.0
Slaughter	>1.5 yrs	307.4±34.7[a]	5.5±1.3[a]	5.9±0.4[a]	72.1±9.7	3.1±1.8[a]
	1.5-3 yrs	280.8±25.1[b]	5.5±1.4[a]	5.8±0.4[a]	75.7±14.0	3.2±2.5[a]
age	3 yrs<	250.2±29.3[c]	3.7±1.6[b]	4.4±1.8[b]	71.9±15.3	1.6±0.6[b]

*,** means significant differences between sex,[a,b,c]means significant differences between slaughter age

According to results, cows had lower carcass weight (247 kg v. 295 kg), EU muscle (3.6 v. 5.5) and fat score (4.2 v. 5.9) than bulls ($P<0.01$). In case of bulls the majority of carcasses were placed to conformation score O+, whereas by cows the most common conformation class was P0. Bulls had higher LT area than cows, however differences were not significant. IMF content was the same for bulls (2.8±1.9) and for cows (2.7±1.9), and slightly lower than the minimum amount (3%) of IMF to achieve acceptable consumer satisfaction. Higher slaughter age significantly decreased hot carcass weight in both sexes. EUROP muscle and fat scores were significantly lower in the highest age group than values obtained in younger slaughter age categories regardless of sex. Older animals produced carcasses with smaller LT area and lower level of IMF ($P<0.001$). Mean collagen content of cows was significantly higher (0.7%), than that of bulls (0.5%).

Collagen (mg/100g) and CT determined connective tissue proportion (%) according to sex:



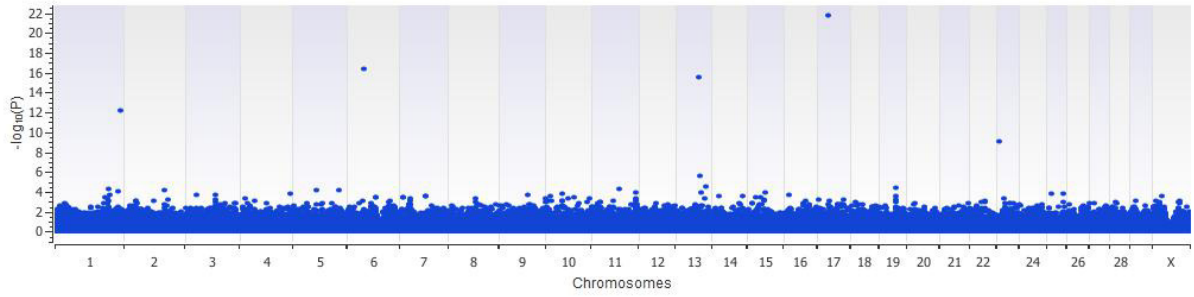
The same tendency could be observed for the CT connective tissue content (0.4% v. 0.7% $p<0.05$). The highest collagen content was measured in the oldest age group (0.65±0.11 mg/100g), which was followed by the intermediate age group (0.59±0.09 mg/100g). The lowest collagen content was obtained in the youngest slaughter age group (>1.5 years). Similar tendency has been observed for CT connective tissue proportion (>1.5yrs: 0.37±0.25%, 1.5-3 yrs:0.49±0.15%, 3yrs<:0.62±0.29%).

Concerning genetic examinations DNA extraction was performed from sperm, blood and in some cases from muscle samples collected from bulls at slaughtering. SNP typing was performed on high-resolution SNP chips developed for cattle (Illumina Bovine HD Chip).

For data screening and identification of loci associated with IMF genomic relationship matrix and multi-locus mixed-model were used to handle covariate effects. Statistical analyses were performed by using the SVS software, which is suitable to fit standard linear models, accounting for population stratification or environmental interaction.

Three loci have been identified to be highly associated with IMF. These loci ($-\log_{10}P>12$) seem to be useful in selection programs and are located on chromosome 6, 13 and 17, their minor allele frequencies are 0.221, 0.162 and 0.106, respectively. IMF values collected from the database has ranged from 0.5 to 5.8 %. Animals with IMF value higher than 3.4 % started to display minor alleles in heterozygous or homozygous form.
Samples were excluded from analysis if call rate was below 95 %. Only SNPs having consistently high call rate (>95%) were included in this study. Duplicated samples (IBD > 0.95) were excluded from dataset. Statistical analyses were performed by SVS software (GoldenHelix, USA). For correction of population structure, genomic kinship matrix has been used in multi-locus mixed model. Phenotypic values have been left as they were, a continuous variable.
The used model was: $y = X\beta + Zu + e$, where y is the IMF, X is the matrix of fixed effects composed of SNPs and covariates (age and sex), Z is the matrix of random animal effects, e

means the residual effects. β and u are vectors representing coefficients of fixed and random effects, respectively.



Manhattan plot of SNPs regarding IMF:
Loci on chromosome 6, 13 and 17 display the highest -$\log_{10}$P values (see dots >14), which are associated with the intramuscular fat content in Hungarian Simmental cattle.

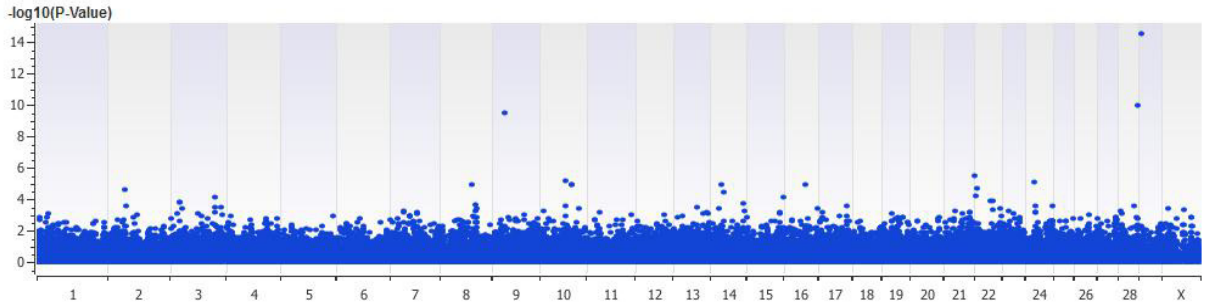
At present breeding decisions in AHSCB are based mainly on breeding values. Estimated breeding values (EBV) signify level of the breeding potential of animals for specific traits. Indexes are scores of genetic merit combining the relative economic values of several EBV traits. Indexes take into account performance data collected on known relatives, relationships between performance traits and degree to which traits are inherited from one generation to the next.

Currently there is no direct method for the estimation of breeding value of fertility (BVF) by bulls. In this case fertility of female offsprings is evaluated. Progeny testing is performed by considering number of inseminations for the successful conception and non-return rate of heifers until 56 days (NR56).
Evaluation of breeding value of beef (BVB) in dual purpose Hungarian Simmental bulls is based on net weight gain, lean meat % and EUROP conformation score of carcasses. Weighting of breeding values is quoted as follows: 22% for net weight gain, 39% for lean meat production and 39% for EUROP conformation score of carcass muscularity.

In both cases (BVF and BVB) incorporation of new markers into the evaluation process of BVF and BVB by AHSCB in current and future breeding programs would by of major importance.

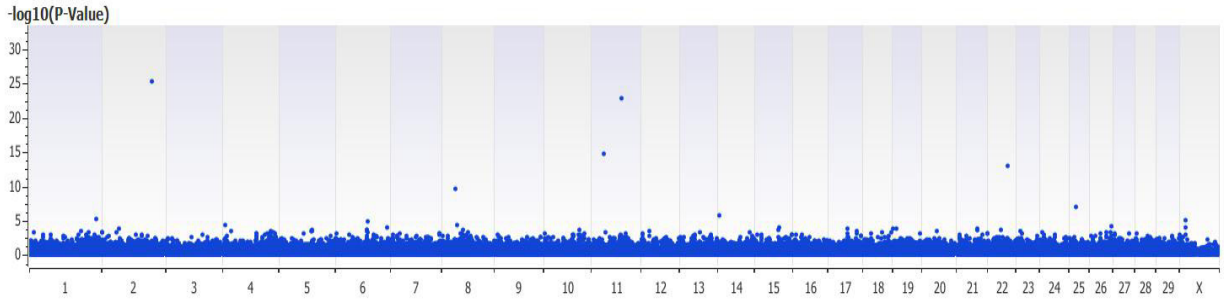
For data screening and identification of loci associated with BVF and BVB, multi-locus mixed-models were used. Statistical analyses were performed by SVS software (GoldenHelix, USA). Phenotypic values (BVB and BVF) have been left as they were, a continuous variable. Genomic inflation factor, lambda value was calculated from the median of the distribution of the chi-square statistic from results divided by the median of the corresponding (ideal) chi-square distribution. Lambda values were 1.06 and 1.09 in case of BVF and BVB, respectively. For correction of population structure, genomic kinship matrix has been used in multi-locus mixed model.
The used model was: $y = X\beta + Zu + e$, where y is the BVB or BVF, X is the matrix of fixed effects composed of SNPs and covariates (age and farm), Z is the matrix of random animal effects. e means the residual effects. β and u are vectors representing coefficients of fixed and random effects, respectively.

All data formatting, filtering and calculations were performed by SVS (GoldenHelix, US) software.
In case of breeding value of fertility (BVF), association analysis was carried out to find correlation between SNP genotypes and breeding parameters data of bulls. Three loci showed considerable association with BVF on chromosome 9, 28 and 29, ($-\log_{10}P$=9.53, 9.94 and 14.55) respectively. Frequencies of their minor alleles are 0.375, 0.355 and 0.354.



Manhattan plot of SNPs regarding BVF:
Loci on chromosome 9, 28 and 29 display the highest $-\log_{10}P$ values (see dots > 8), which are associated with the breeding value of fertility in Hungarian Simmental cattle.

According to the analysis outcome, several loci have been identified to be associated with breeding value of beef. Among seven loci ($-\log_{10}P$ >5) two seem to be useful in selection program, which are located on Chromosome 2 and 11. Breeding values -collected from database- ranged from 80 to 130. Allelic pattern of locus at Chr 2 has changed to homozygous in all animals with BV higher than 110. Majority of animals with BV lower than 102 were homozygous at the locus on Chr 11. Their minor allele frequencies (0.438 and 0.229) provide straightforward possibility to assist selection by molecular tools.



Manhattan plot of SNPs regarding BVB:
Loci on chromosome 2 and 11 display the highest $-\log_{10}P$ values(see dots > 20), which are associated with the breeding value of beef in Hungarian Simmental cattle.

It seems reasonable to conclude that it was no relationship between EUROP classification of carcasses and IMF content of LD, yet in contrast to the fact that IMF was highly correlated to CT data. Intramuscular fat was affected by slaughter weight and was negatively correlated with growth rate ($r = -0.40$ to -0.49).

Proportion of IMCT in LD increased with slaughter age and older cows had higher collagen and connective tissue proportion than bulls. Mixed CT scans can be used for the analysis of IMCT content.

SNPs and their positions found in this study are different from other hits described in other breeds and studies. Molecular tests can provide facilities for direct selection among alleles of highlighted SNPs, however benefits of given alleles depend on the breed itself and on economic goals. We propose to utilise selection for favourable alleles at reported loci on chromosomes 1, 6, 13 and 17, if increased IMF is desirable.
A possible marker assisted selection approach -such as selecting for favourable alleles at reported loci on chromosome 9, 28 and 29 might be performed, if higher fertility is desirable.
To increase weight gain and lean meat production we recommend selection for favourable alleles at reported loci on chromosomes 2 and 11.
We suggest incorporation of described results by AHSCB into the evaluation process of BVF and BVB in current and future breeding programs.
Results presented above have been submitted to peer-reviewed journals for publication.