

Thesis of Doctoral (Ph.D.) Dissertation

**GENOME WIDE ASSOCIATION STUDY AND
COMPLEX ANDROLOGICAL
EXAMINATION OF HUNGARIAN SWINE
BREEDS IN ORDER TO IMPROVE
REPRODUCTIVE PARAMETERS**

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1. BACKGROUND OF THE DOCTORAL DISSERTATION

Pig breeding is one of the most important sectors of Hungarian animal husbandry. According to December 2019 data, the pig population in Hungary was 2,6 million pigs (KSH, 2020a). Per capita consumption of pork showed a continuous increase between 2004 and 2018, according to the latest KSH (2020b) data: in 2004 the per capita consumption of pork was: 25.9 kg, in 2018: 32.9 kg.

Successful breeding programs can add to the profitability of breeders and well-planned programs can also be very rewarding to those developing and implementing them.

Further development of the porcine sector is an important task, both economically and in terms of breeding. Reserves in this area are mainly related to the improvement of reproduction results of the stocks and the reduction of additional costs.

The Hungarian breeds (including Hungarian Large White and Hungarian Landrace) have many advantageous characteristics. They have good adaptability and stress tolerance as well as good production properties. Their positive traits were recognized at the beginning of their breeding, so these breeds are still used as paternal (Hungarian Landrace) and maternal partners (Hungarian Large White) as well. Nevertheless, in recent decades Western European hybrids have achieved better production results compared to Hungarian Large White and Hungarian Landrace breeds, thus pushing these two breeds out of the forefront.

Therefore, it is a very important task to preserve the Hungarian breeds and increase their competitiveness. They lag behind the crossed breeds primarily in reproductive characteristics, which can cause considerable economic handicap to the breeder.

Targeted genetic testing, combined with long-term and consistent selection work, can contribute to the improvement of the reproductive parameters and competitiveness of the Hungarian breeds.

1. 1. Aims of the research

The main aims of my dissertation were as follows:

- andrological and spermatological examination of Hungarian Large White and Hungarian Landrace boars:
 - motility (Sperm Class Analyzer – SCA, Microptic S. L., Spain), ultrasonography (Tringa Linear VET, Esaote, Spain), thermography (InfiRay IRAC200H, Yantai IRay Technology Co., Ltd., China) and morphology: Kovacs-Foote staining procedure to assess the suitability of the ejaculate for fertilization and the health status of the male genitals

- investigation concerning the reliability of measurements obtained by the portable Ongo device at Hungarian Large White, Hungarian Landrace, Danbred × Duroc, Duroc × Pietrain breeds

- investigation of single-nucleotide polymorphisms (SNPs) in females (Hungarian Large White) associated with the following reproductive parameters:
 - interval between litters (IBL)
 - total number of piglets born (TNB)
 - litter weight of piglets born alive (LWA)
 - number of piglets born alive (NBA)
 - number of piglets born dead (NBD)
 - average litter weight on the 21st day (M21D)
 - growth rate (GR)
 - number of litters (NL)
 - percentage of litters (PL)
 - mean number of piglets born alive (MNBA)
 - mean number of piglets born dead (MNBD)
 - mean total number of piglets born (MTNB)

2. MATERIAL AND METHODS

2. 1. Genetic examinations

2. 1. 1. Collection of samples

Blood samples were collected from 300 individuals of eleven farms, which were stored at -20°C in the NARIC-RIABNMS genetic laboratory until DNA extraction. The samples were selected considering the following criteria: to represent individuals with low and high values regarding reproductive properties; the samples should represent the whole population; there should be no relatedness between the selected individuals.

2. 1. 2. Identification of SNP using microarray

Genotyping was performed by Neogen Europe Ltd. (Scotland, UK) using Illumina Porcine SNP60K BeadChip (GeneSeek® Genomic Profiler™ High-Density) which contained 61.177 SNPs.

Reproductive and breeding data required for the study were furnished by the Hungarian Purebred Pig Breeders' Association.

2. 2. Evaluation of genotyped data

Samples were included in the study if the call rate was above 95%. Duplicated samples (Identity By Descent, IBD > 0.95) and loci with a MAF < 0.05 were excluded from the dataset. The final database included 290 animals and 56,592 SNPs. During the genotyping multi-locus mixed models were used to explore the loci associated with the reproductive parameters and screening the data. Phenotypic values were left as they were, a continuous variable. The genomic inflation factor (λ) was calculated from the median of the distribution of the chi-square (χ^2) statistic from results divided by the median of the corresponding (ideal) χ^2 distribution (ARMITAGE, 1955). For structural correction of the population, genomic kinship matrix was used in a multi-locus mixed model (SEGURA et al., 2012).

SVS software (GoldenHelix, USA) was used for data formatting, statistical analysis, and data filtering. SNPs were identified on chromosomes that were associated with reproductive properties using Manhattan plot.

2. 3. Andrological and spermatological examinations

The blood and sperm samples were collected from 21 males of four Hungarian farms. The gloved-hand method (BASURTO-CUBA and EVANS 1981; ALTHOUSE et al., 2015) was used for sperm collection. During sperm collection, ultrasound system (Tringa Linear VET, Esaote, Spain) and thermography camera was applied (InfiRay IRAC200H, Yantai IRay Technology Co., Ltd., China) to assess the morphological condition of the testis and to examine the thermoregulation of the testis. Evaluation of the ultrasound images was performed with GIMP (GNU Image Manipulation Program, 2.10.10) and the analysis of the thermal images was performed with FLIR thermal imaging evaluation program.

Table 1

The number of collected samples and examinations at farms

		farms			
		A	B	C	total
blood samples (n)	HLW	3	3	-	11
	HL	4	1		
ejaculates (n)	HLW	3	3	3	16
	HL	4	1	2	
ultrasonography	HLW	3	-	5	12
	HL	4			
CASA	HLW	3	3	3	16
	HL	4	1	2	
thermography	HLW	3	-	5	12
	HL	4			

HLW (Hungarian Large White)

HL (Hungarian Landrace)

Table 1 shows the collected blood and sperm samples and the number of examinations on the farms. In some cases, I did not have the opportunity to perform all examinations (blood sampling, ultrasonography and thermography - squares left blank). I have previously performed tests on other pig breeds in order to practice the testing techniques.

2. 4. Motility and morphological examinations

Laboratory analyses were performed in the National Agricultural Research and Innovation Centre-Research Institute for Animal Breeding, Nutrition and Meat Science (NARIC-RIABNMS). The ejaculates were stored at 16°C after sampling. The spermatozoa were evaluated by the KOVÁCS-FOOTE (1992) staining method. This method shows live and dead spermatozoa, acrosome status, and cell abnormalities. During the staining procedure, Chicago sky blue 6B (CSB) solution (Sigma-Aldrich C8679, Sigma Aldrich Corporation, St. Louis, MO, United States) was used to stain live and dead spermatozoa, 0.2% neutral red (Sigma Aldrich N-7005, Sigma Aldrich Corporation, St. Louis, MO, United States) was used to fix the samples and 1N HCL solution containing 5% formaldehyde (37% formaldehyde solution), 7.5% Giemsa solution (Sigma G S500, Sigma Aldrich Corporation, St. Louis, MO, United States) for acrosome staining. Before sperm staining, the fresh samples were diluted with buffered NaCl solution (0.06% K₂HPO₄ anhydrate and 0.825% NaCl) and formalin citrate (2.9% Na₃ citrate and 0.1% formalin).

Motility of sperms was analyzed with computer assisted sperm analysis program (CASA, AndroVision, Minitüb, Germany) immediately after transporting the samples to the institute. Samples were further diluted to the required concentration (60x10⁶ sperm/ml), the samples were further diluted with Beltsville Thawing Solution (BTS) in a ratio of 1:15 (v/v). The program records video from 10 areas, analyses them and collects data. The cells were classified into three categories (non-motile, non-progressive motility, progressive motility). To analyze the type of movement, adjustment parameters for pigs were used from the CASA user manual: non-motile: AOC (average orientation change) <2.5; TM%: AOC > 2.5 and DSL (distance straight line) <4.5 µm /s; PM%: AOC > 2.5 and DSL > 4.5 µm /s (HORVÁTH, 2015).

2. 3. Monitoring the suitability of a portable motility analyzer

There were collected total of 581 ejaculates from eight males (two Hungarian flat, two Hungarian great white, two duroc × pietrain, two danbred × duroc). Freshly collected ejaculates were diluted with Beltsville Thawing Solution (BTS) (PURSEL and JOHNSON, 1975) and then stored at 16°C and transported to NARIC-RIABNMS. The 581 ejaculates included in the study were diluted to the concentration required for motility tests (50 x10⁶ sperm/ml), followed by both desktop CASA software (Sperm Class

Analyzer - SCA, Microptic SL, Barcelona, Spain) and a mobile CASA (Ongo) device (Ongo Sperm Test ®, Microfluidlabs, Budapest, Hungary) for concentration, motility and progressive motility.

The examination method was similar in case of both devices (Microptic, Ongo) for all the 581 ejaculates. Classification of spermatozoa was analogous to the protocol suggested by BUSS et al., (2019): spermatozoa with average orientation change $<8 \mu\text{m}$ were considered immotile; those with curvilinear velocity $\geq 10 \mu\text{m/s}$, distance straight line $\geq 6 \mu\text{m}$ and radius $\geq 15 \mu\text{m}$ were considered progressively motile.

Ongo chamber slides were used to examine the samples with both devices. The application of both systems was preceded by the setting of different parameters such as animal species, lens zoom, type of extender.

Statistical analysis was performed by Bland-Altman method (BLAND and ALTMAN, 1986). The method describes the average differences between desktop and portable CASA measurement techniques: concentration, motility, and progressive motility are compared. A difference of $\pm 20\%$ between methods is considered acceptable in clinical andrology studies. This method of comparison was considered by MORTIMER et al., (2015) as a “gold standard” sperm analysis method where upper and lower 95% limits of agreement were calculated.

3. RESULTS

3. 1. Identification of SNPs

The aim of the genetic examination was to identify SNPs associated with the twelve examined reproductive parameters (interval between litters, total number of piglets born, litter weight of piglets born alive, number of piglets born alive, number of piglets born dead, average litter weight on the 21st day, growth rate, number of litters, percentage of litters, mean number of piglets born alive, mean number of piglets born dead, mean total number of piglets born). Several SNPs were found to be associated with five different parameters. Nearby genes of the identified SNPs were located by *Sus scrofa* Assembly Build 11.1 database.

3. 1. 1. Total number of piglets born

At the examined 290 Hungarian Large White sows, three SNPs were identified to be associated with the total number of piglets born (TNB). These loci are located on chromosomes 1, 6, and 13 (Table 2, Figure 1).

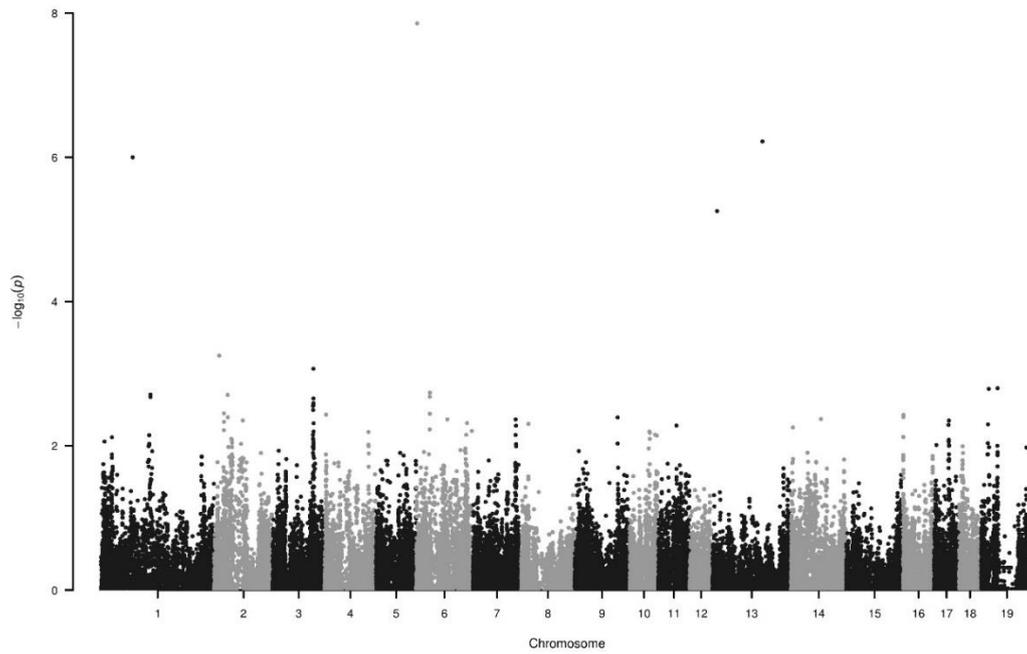
Table 2

List of loci associated with the total number of piglets born (TNB), their genomic location and nearest genes

Marker ss ID	Chr:position	$-\log_{10}P$	candidate gene(s) near the marker	MAF	FDR
rs80878088	1:88143914	6.00	<i>RFPL4B</i> , <i>MARCKS</i> ,	0.298	0.016
s336610321	6:2594634	7.86	<i>FBXO31</i> <i>FOXL1</i> , <i>MTHFSD</i>	0.299	6.88e-4
rs326153933	13:139009753	6.22	<i>FGF12</i>	0.364	0.015

MAF (minor allele frequency); FDR (false discovery rate)

Figure 1: Manhattan plot of SNPs associated with the total number of piglets born



Loci on chromosomes 1, 6 and 13 display the highest $-\log_{10} P$ values (see dots >6) which are associated with the total number of piglets born. Number 19 on horizontal axis refers to chromosome X.

3. 1. 2. Litter weight of piglets born alive

Seven loci were identified to be associated with the litter weight of piglets born alive (LWA) examined 290 Hungarian Large White sows. These loci are located on chromosomes 5, 6, 14, 16, 17, X (Table 3, Figure 2).

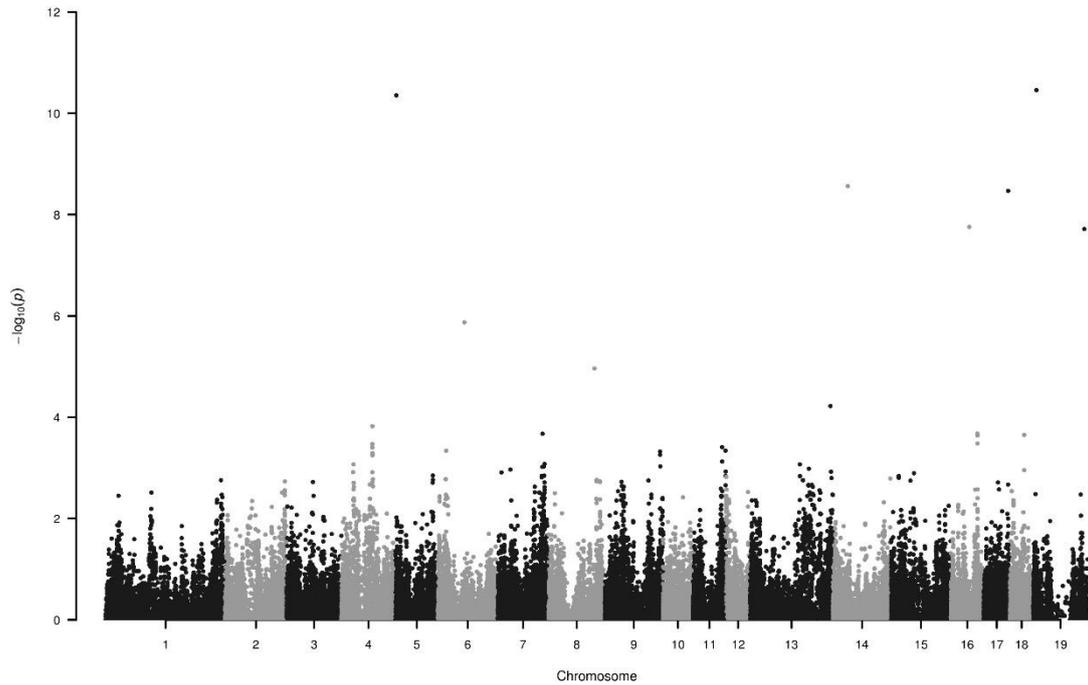
Table 3

List of loci associated with the litter weight of piglets born alive (LWA), their genomic location and nearest genes

Marker ss ID	Chr:position	$-\log_{10}P$	candidate gene(s) near the marker	MAF	FDR
rs81382693	5:1912703	10.35	<i>ARHGAP8</i> , <i>PRR5</i>	0.425	1.10e-06
rs340060083	6:70048043	5.87	<i>PADI2</i> , <i>PADI1</i>	0.397	9.49e-03
rs345681434	14:39399038	8.56	<i>MED13L</i> , <i>TBX3</i>	0.115	4.53e-05
rs81459332	16:48711236	7.76	<i>ERBB2IP</i>	0.155	1.74e-04
rs80882327	17: 57391800	8.47	<i>BMP7</i>	0.492	4.22e-05
rs81473286	X:8718698	10.46	<i>AMELX</i> , <i>ARHGAP6</i>	0.446	1.73e-06
rs319594780	X:135147279	7.72	<i>SLITRK</i> cluster	0.348	1.59e-04

MAF (minor allele frequency); FDR (false discovery rate)

Figure 2: Manhattan plot of SNPs associated with the litter weight of piglets born alive



Loci on chromosomes 5, 14, 16, 17 and X display the highest $-\log_{10} P$ values (see dots >5) which are associated with the litter weight of piglets born alive. Number 19 on horizontal axis refers to chromosome X.

3. 1. 3. Number of piglets born dead (NBD)

In the examined Hungarian Large White sows (290), seven loci were found to be associated with the number of piglets born dead (NBD) which are located on chromosomes 5, 6, 13, 14, 15, 16,18 (Table 4, Figure 3).

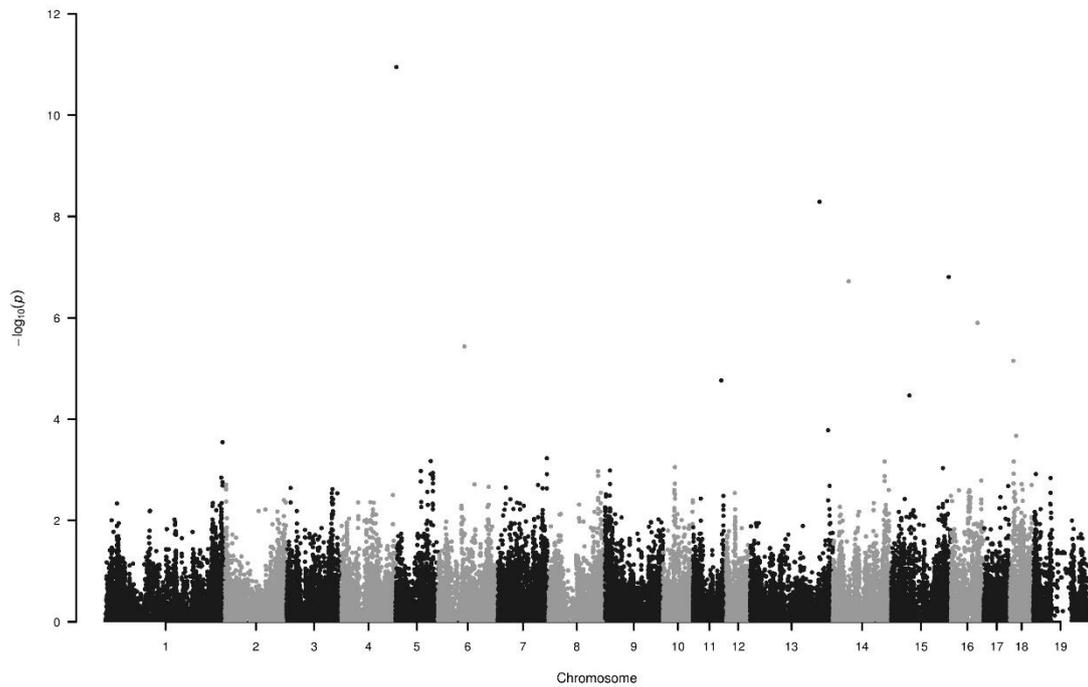
Table 4

List of loci associated with the number of piglets born dead (NBD), their genomic location and nearest genes

Marker ss ID	Chr:position	$-\log_{10}P$	candidate gene(s) near the marker	MAF	FDR
rs81382693	5:1912703	10.95	<i>ARHGAP8</i>	0.425	5.56e-07
rs340060083	6:70048043	5.43	<i>PADI2, PADI1</i>	0.397	3.03e-02
rs80893810	13:183254699	8.29	<i>CADM2, LIPI SNORA70</i>	0.335	1.27e-04
rs80845657	14:41396206	6.72	<i>RPL6, TBX3</i>	0.095	2.35e-03
rs329723588	15:152057161	6.81	<i>SCLY</i>	0.090	2.58e-03
rs338594773	16:70502947	5.90	<i>EBF1</i>	0.365	1.24e-02
rs333328959	18:8927486	5.15	<i>BRAF, MKRN1, PPAR</i>	0.069	4.99e-02

MAF (minor allele frequency); FDR (false discovery rate)

Figure 3: Manhattan plot of SNPs associated with the number of piglets born dead



Loci on chromosomes 5, 6, 13, 14, 15, 16 and 18 display the highest $-\log_{10} P$ values (see dots >5) which are associated with the number of piglets born dead. Number 19 on horizontal axis refers to chromosome X.

3. 1. 4. Average litter weight on the 21st day

The only locus found to be associated with the average litter weight on the 21st day (M21D) in the examined Hungarian Large White sows (290) is located on chromosome 1 (Table 5, Figure 4).

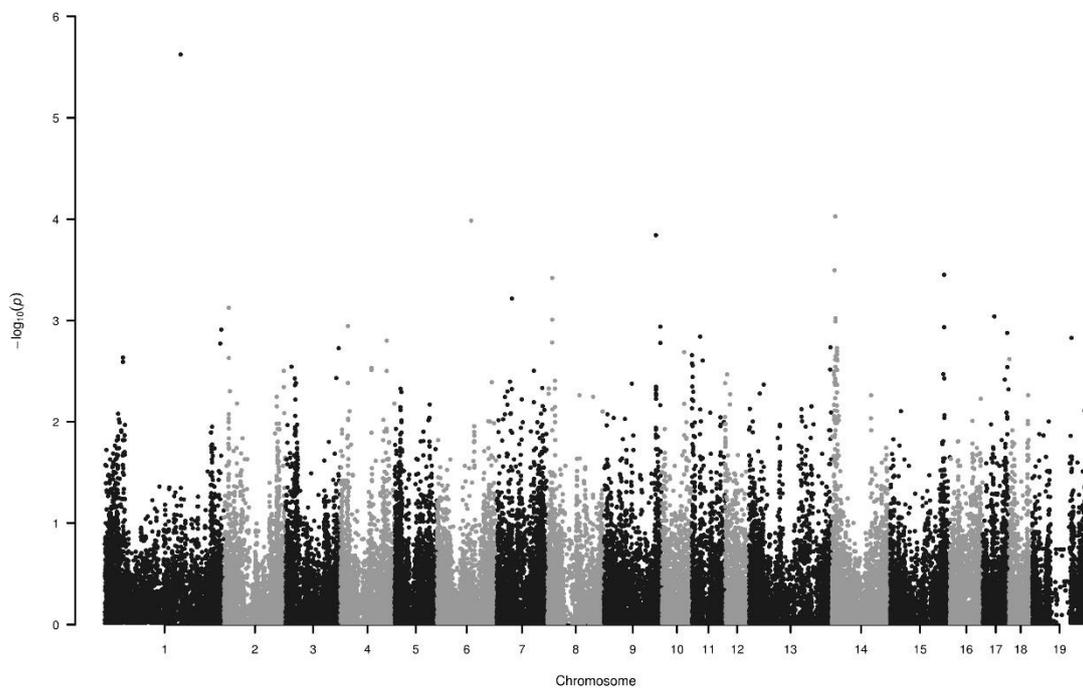
Table 5

The locus associated with the average litter weight on the 21st day (M21D), its genomic location and nearest genes

Marker ss ID	Chr:position	$-\log_{10}P$	candidate gene(s) near the marker	MAF	FDR
rs699316219	1:200350940	5.62	<i>ARF6</i> , <i>ABHD12B</i>	0.461	0.117

MAF (minor allele frequency); FDR (false discovery rate)

Figure 4: Manhattan plot of the SNP associated with the average litter weight on the 21st day



The SNP is located on chromosome 1 and displays the highest $-\log_{10} P$ value (see dot >5). Number 19 on horizontal axis refers to chromosome X.

3. 1. 5. Interval between litters

In the examined 290 Hungarian Large White sows, one SNP was found to be associated with the interval between litters (IBL) which is located on chromosome 8 (Table 6, Figure 5).

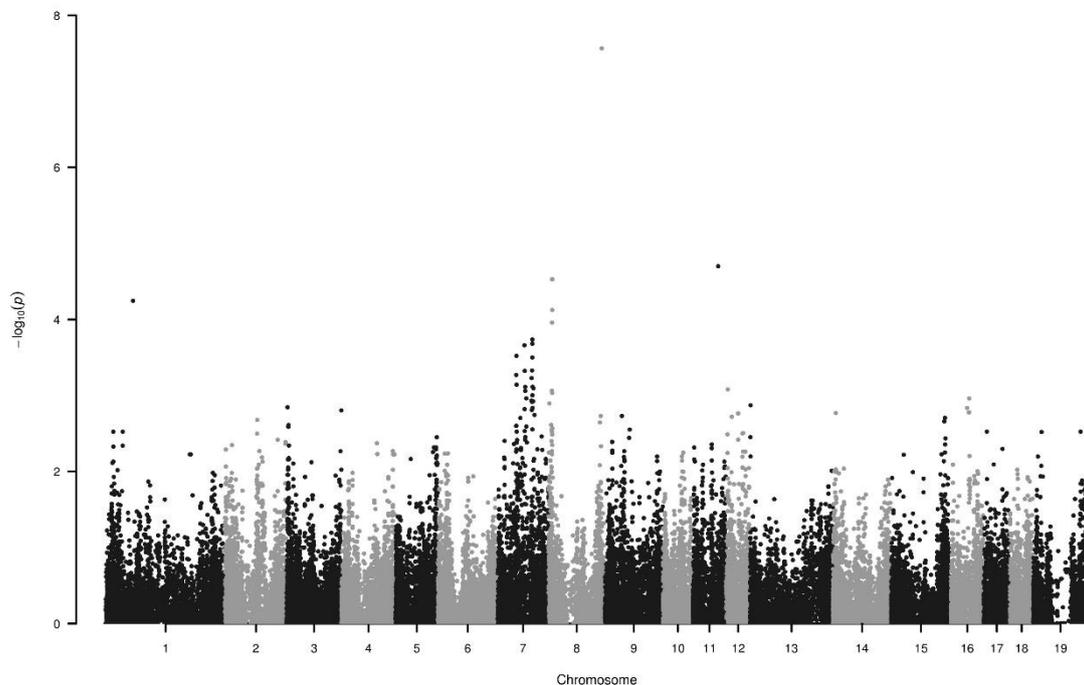
Table 6

The locus associated with the interval between litters (IBL), its genomic location and nearest genes

Marker ss ID	Chr:position	$-\log_{10}P$	candidate gene(s) near the marker	MAF	FDR
rs81301813	8:140274549	7.56	<i>PKD2</i> , <i>SPP1</i> , <i>MAPK10</i>	0.001	1.35e-03

MAF (minor allele frequency); FDR (false discovery rate)

Figure 5: Manhattan plot of SNP associated with the interval between litters



The SNP is located on chromosome 8 and displays the highest $-\log_{10} P$ value (see dot >7). Number 19 on horizontal axis refers to chromosome X.

3. 2. Evaluation of andrological and spermatological examinations

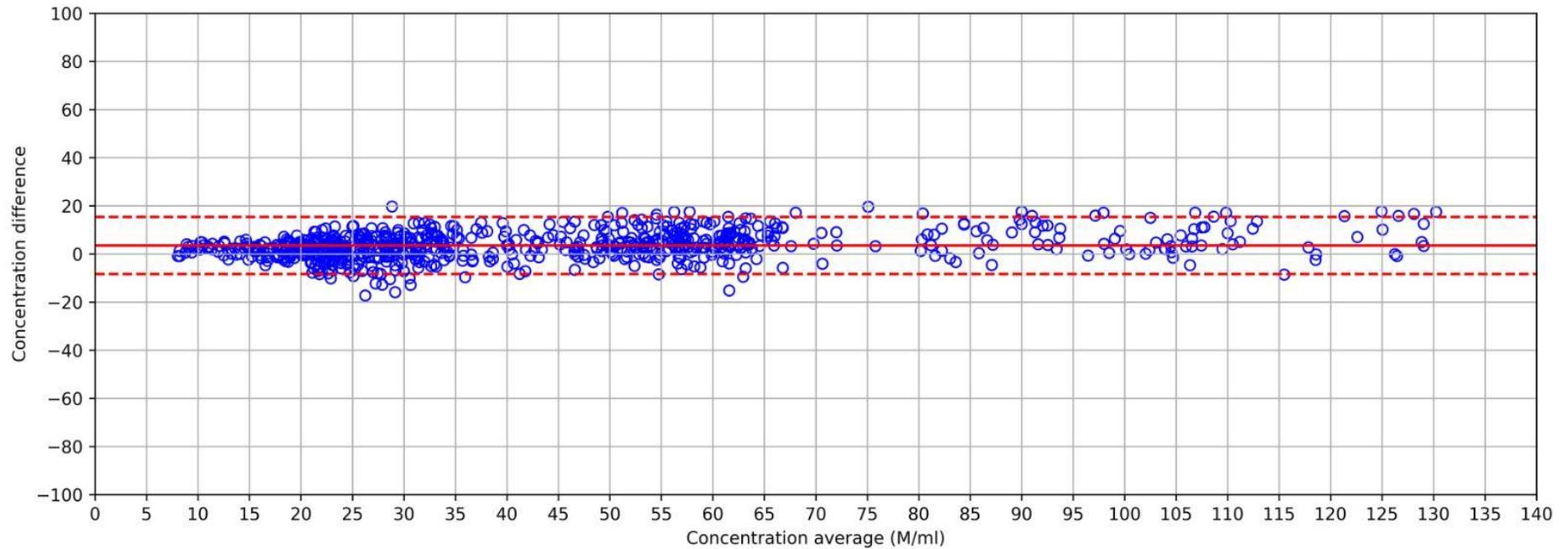
The aim of the ultrasound, thermographic examination and Kovacs-Foote staining procedure was to assess the suitability of the ejaculate for fertilization and the health status of the male genitals. With functional testing (CASA) and monitoring of testicular tissue structure and cell integrity, we sought to apply the widest possible methodology. In case of males, in a similar way to females, it is possible to perform molecular genetic examinations for the improvement of reproductive parameters.

Following the test of the reproductive organs of boars no serious abnormalities were found by thermography in scrotal thermoregulation or the testicular tissue by ultrasonography. In the examined ejaculates there were no motility abnormalities (CASA). Concerning dead and live spermatozoa (Kovacs-Foote staining method) there were not extreme abnormalities detected.

3. 3. Monitoring the suitability of a portable motility analyzer

During the comparative study of the two devices a total of 1,162 measurements (581 data pairs) were performed. The results of the two instruments were compared using Bland-Altman plot for concentration, motility, and progressive motility (Figures 6, 7, and 8). The mean differences of concentration, motility, and progressive motility were within the calculated 95% limits of agreement ($d \pm 2SD$) with strong agreement between the two methods (Ongo and Microptic CASA).

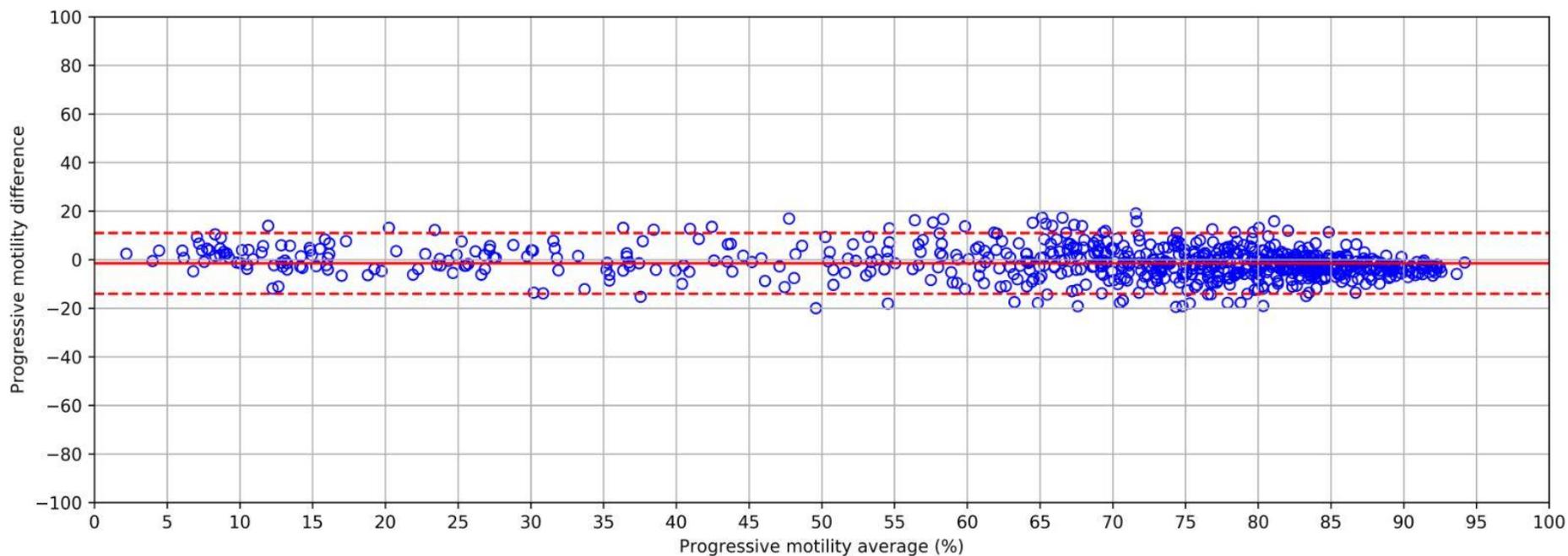
Figure 6: Bland Altman plot: Concentration [M/ml] agreement of Desktop (Microptic) CASA vs Ongo Sperm Analyzer



Red line (mean differences of concentration): -3.85 M/ml. Dashed, red lines 95% limits of agreement ($d \pm 2SD$) - lower: -8.28 and upper: 15.41. On the graph the X axis shows the average of the measurements of the two devices (M/ml) and the Y axis represents the difference between Ongo and Microptic CASA paired measurements (M/ml).

In terms of concentration, the Bland-Altman diagram showed an acceptable agreement between Ongo and Microptic CASA.

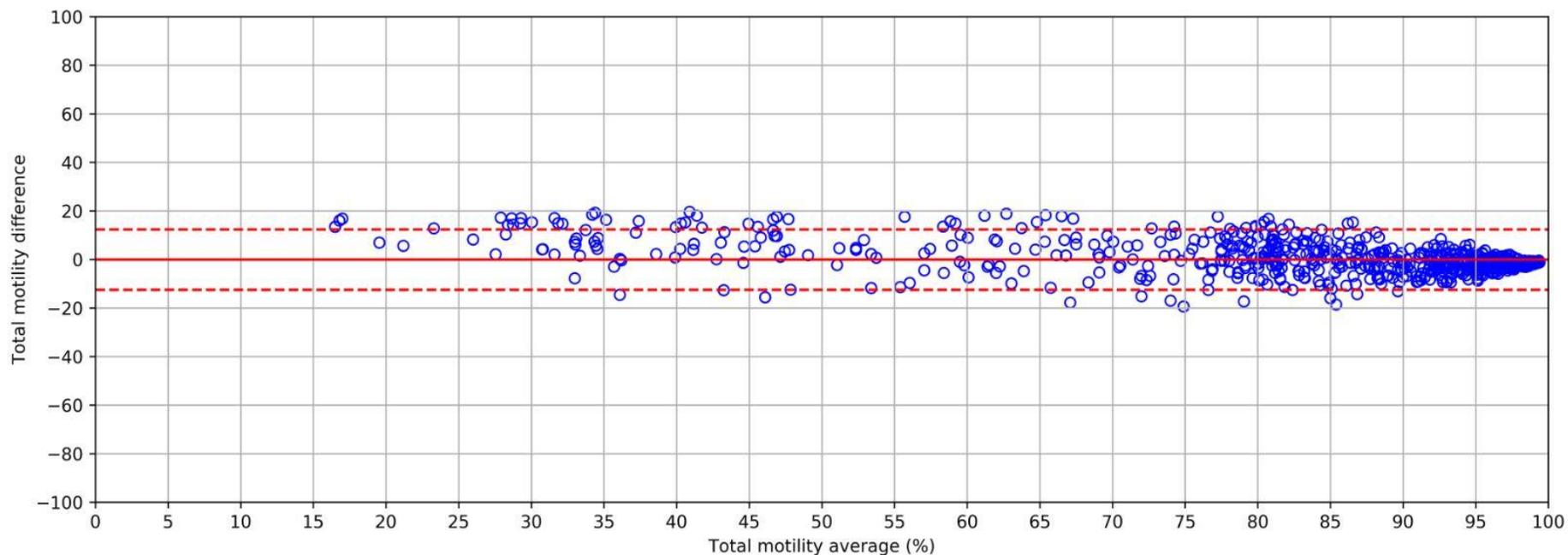
Figure 7: Bland Altman plot: Progressive motility [%] agreement of Desktop (Microptic) CASA vs Ongo Sperm Analyzer



Red line (progressive motility mean differences): 1.09%. Dashed, red line: 95% limits of agreement ($d \pm 2SD$) - lower: -13.98% and upper: 11%. On the graph the X axis shows the average of the measurements of the two devices (%) and the Y axis represents the difference between Ongo and Microptic CASA paired measurements (%).

Based on the results, the two instruments also showed an acceptable similarity in terms of progressive motility.

Figure 8: Bland Altman plot: Total motility [%] agreement of Desktop (Microptic) CASA vs Ongo Sperm Analyzer



Red line (motility mean differences): -0.91%. Dashed, red line: 95% limits of agreement ($d \pm 2SD$) - lower: - 2.43% and upper: 12.42%.

On the graph the X axis shows the average of the measurements of the two devices (%) and the Y axis represents the difference between Ongo and Microptic CASA paired measurements (%).

In terms of motility, the Bland-Altman analysis showed an acceptable agreement between the examined devices.

4. NEW SCIENTIFIC RESULTS

1. **Three SNPs** (marker ss ID: rs80878088, rs336610321, rs326153933) on chromosomes 1, 6 and 13 were identified to be associated ($-\log_{10} P = 6.0, 7.86$ and 6.22) with the total number of piglets born (TNB) of Hungarian Large White sows.
2. **Seven SNPs** (marker ss ID: rs81382693, rs340060083, rs345681434, rs81459332, rs80882327, rs81473286, rs319594780) on chromosomes 5, 6, 14, 16, 17 and X were identified to be associated ($-\log_{10} P = 10.35, 5.87, 8.56, 7.76, 8.47, 10.46, 7.72$) with the litter weight born alive (LWA) of Hungarian Large White sows.
3. **Seven SNPs** (marker ss ID: rs81382693, rs340060083, rs80893810, rs80845657, rs329723588, rs338594773, rs333328959) on chromosomes 5, 6, 13, 14, 15, 16 and 18 were identified to be associated ($-\log_{10} P = 10.95, 5.43, 8.29, 6.72, 6.81, 5.90$ and 5.15) with the number of piglets born dead (NBD) of Hungarian Large White sows.
4. **One SNP** (marker ss ID: rs699316219) on chromosome 1 was identified to be associated ($-\log_{10} P = 5.62$) with the average litter weight on the 21st day (M21D) of Hungarian Large White sows.
5. **One SNP** (marker ss ID: rs81301813) on chromosome 8 was identified to be associated ($-\log_{10} P = 7.56$) with the interval between litters (IBL) of Hungarian Large White sows.
6. Strong correlation was found between the investigated (Microptic and Ongo) devices: Bland-Altman plot (concentration: -3.85 M/ml; progressive motility: 1.09% ; total motility: -0.91%) at Hungarian Large White, Hungarian Landrace, Danbred \times Duroc, Duroc \times Pietrain swine breeds.

5. PRACTICALLY APPLICABLE RESULTS

1. By identifying the 19 single-nucleotide polymorphisms associated with the examined reproductive parameters, it is possible to further improve the performance of our Hungarian Large White and Hungarian Landrace breeds. In this way, there is possible to breed selected Hungarian breeds in accordance with modern market and economic expectations. Breeding systems based on selected Hungarian breeds for higher genetic potential provide an opportunity for stock replacement within our borders, reducing even the possibility of “naturalization” of some diseases that do not yet occur in Hungary.
2. The economic importance of the high breeding potential in the pig sector confirms the most accurate knowledge regarding fertility of boars. In order to develop artificial insemination, an objective, fast and cost-effective method is needed to assess the quality of sperm and predict its fertility. The fertility of an individual is determined by the results of a combination of ejaculate morphology and motility analysis. During motility testing, a number of properties, including concentration and progressive motility, can be analysed using a computer assisted phase contrast microscopy, which is a commonly used laboratory device. The Ongo appliance is a portable version of desktop CASA systems with a user-friendly interface and software. It can be easily used even without professional qualifications. The tool is a practical and cost-effective option if no infrastructure, material and/or human resources are available for a desktop CASA system. It is a fast and accurate instrument for sperm analysis in animal husbandry for objective examination of ejaculate as an alternative to the desktop CASA device.

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7. LIST OF PUBLICATIONS



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Registry number: DEENK/352/2020.PL
Subject: PhD Publication List

Candidate: Eszter Erika Balogh
Doctoral School: Doctoral School of Animal Husbandry
MTMT ID: 10055754

List of publications related to the dissertation

Hungarian scientific articles in Hungarian journals (2)

1. **Balogh, E. E.**, Dálnoki, A. B., Rózsa, L., Rátky, J., Zsolnai, A., Anton, I.: Magyar nagyfehér kocák szaporasági értékmérőivel kapcsolt szelekciós markerek azonosítása.
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2. **Balogh, E. E.**, Dálnoki, A. B., Rózsa, L., Rátky, J., Zsolnai, A., Anton, I.: Hazai és nemzetközi szaporodásbiológiai és genetikai kutatások kronológiai áttekintése a sertéságazatban (Irodalmi áttekintés).
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ISBN: 9786155403101

Total IF of journals (all publications): 2,671

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