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**Title:** Genome-wide association study for frozen-thawed sperm motility in stallions across various horse breeds

**Type of Manuscript:** Article

**Abstract**

Objective: The semen quality of stallions including sperm motility is an important target of selection as it has a high level of individual variability. However, effects of the molecular architecture of the genome on the mechanisms of sperm formation and their preservation after thawing have been poorly investigated. Here, we conducted a genome-wide association study (GWAS) for the sperm motility of cryopreserved semen in stallions of various breeds. Methods: Semen samples were collected from the stallions of 23 horse breeds. The following semen characteristics were examined: progressive motility (PM), progressive motility after freezing (FPM), and the difference between PM and FPM. The respective DNA samples from these stallions were genotyped using Axiom™ Equine Genotyping Array. Results: We performed a GWAS search for single nucleotide polymorphism (SNP) markers and potential genes related to motility properties of frozen-thawed semen in the stallions of various breeds. As a result of the GWAS analysis, two SNP markers, rs1141327473 and rs1149048772, were identified that were associated with preservation of the frozen-thawed stallion sperm motility, the relevant putative candidate genes being NME8, OR2AP1 and OR6C4. Potential implications of effects of these genes on sperm motility are herein discussed. Conclusion: The GWAS results enabled us to localize novel SNPs and candidate genes for sperm motility in stallions. Implications of the study for horse breeding and genetics are a better understanding of genomic regions and candidate genes underlying stallion sperm quality, and improvement in horse reproduction and breeding techniques. The identified markers and genes for sperm cryotolerance and the respective genomic regions are promising candidates for further studying the biological processes in the formation and function of the stallion reproductive system.



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# Genome-wide association study for frozen-thawed sperm motility in stallions across various horse breeds

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## ABSTRACT

**Objective:** The semen quality of stallions including sperm motility is an important target of selection as it has a high level of individual variability. However, effects of the molecular architecture of the genome on the mechanisms of sperm formation and their preservation after thawing have been poorly investigated. Here, we conducted a genome-wide association study (GWAS) for the sperm motility of cryopreserved semen in stallions of various breeds.

**Methods:** Semen samples were collected from the stallions of 23 horse breeds. The following semen characteristics were examined: progressive motility (PM), progressive motility after freezing (FPM), and the difference between PM and FPM. The respective DNA samples from these stallions were genotyped using Axiom™ Equine Genotyping Array.

**Results:** We performed a GWAS search for single nucleotide polymorphism (SNP) markers and potential genes related to motility properties of frozen-thawed semen in the stallions of various breeds. As a result of the GWAS analysis, two SNP markers, rs1141327473 and rs1149048772, were identified that were associated with preservation of the frozen-thawed stallion sperm motility, the relevant putative candidate genes being *NME8*, *OR2AP1* and *OR6C4*. Potential implications of effects of these genes on sperm motility are herein discussed.

**Conclusion:** The GWAS results enabled us to localize novel SNPs and candidate genes for sperm motility in stallions. Implications of the study for horse breeding and genetics are a better understanding of genomic regions and candidate genes underlying stallion sperm quality, and improvement in horse reproduction and breeding techniques. The identified markers and genes for sperm cryotolerance and the respective genomic regions are promising candidates for further studying the biological processes in the formation and function of the stallion reproductive system.

**Keywords:** *Equus caballus*; Stallion; Sperm; Cryopreservation; SNPs; Candidate Genes

## INTRODUCTION

There is a growing interest in the preservation of genetic material from stallions with outstanding phenotypic traits using cryopreservation of spermatozoa [1–3]. Over the past decades, sperm cryopreservation is one of the most widely used methods to preserve biological material in domestic animals (e.g., [4–6]) that is also used as one of gene pool conservation strategies (e.g., [7–9]. However, stallion semen is less resistant to ultra-low temperatures as compared, for example, to bull semen. Importantly, as one of the key targets of selection, sperm quality and cryotolerance in stallions have a high individual variation that depends on both environmental and genetic factors [2,3,10]. To date, horse breeding involves a widespread use of artificial insemination (with the exception of thoroughbred racehorses), and that is why high quality of cryopreserved semen is vital and pivotal [3,11,12].

Genome organization in sperm is functionally instrumental for controlling fertilization and early developmental processes in animals [13–15]. Determination of genetic factors affecting sperm quality indicators and sperm cryotolerance is therefore of great significance, and certain candidate genes have been found to be associated with male fertility traits and sperm quality after thawing. These genes include, for example, testis-sperm specific *FKBP6*, a candidate for impaired acrosome reaction [16,17], *PLCZ1* [18], *CRISP3* [19,20], and some others genomic variants [10,21,22]. Spermatozoa progressive motility measured as speed of forward progression with flagellar movement (see for review [23,24]) is one of the most important semen quality properties before freezing and after thawing. However, the relationship between the molecular architecture of the genome, on the one hand, and mechanisms of sperm formation and their preservation after thawing, on the other, is poorly understood and requires further detailed investigation [25].

In this regard, the aim of the present investigation was to perform a genome-wide association study (GWAS) for genomic variants relevant to sperm motility of cryopreserved semen in stallions across various horse breeds using a high density single nucleotide polymorphism (SNP) chip. As a result,

we were able to identify a few suggestive SNP markers and relevant candidate genes that are worthy of further research and applications in horse breeding.

## MATERIALS AND METHODS

### Animals and sample collection

Sampling procedure was approved by the Russian Research Institute of Farm Animal Genetics and Breeding (RRIFAGB) – Branch of the L. K. Ernst Federal Science Centre for Animal Husbandry (Protocol No. 2020/2), adhered to and performed in accordance with the appropriate ethical guidelines (Law of the Russia Federation on Veterinary Medicine No. 4979-1 dated 14 May 1993). The authors declare that stallion semen samples were properly collected by trained personnel following strict veterinary requirements and keeping animal discomfort and stress to a minimum.

To conduct the present GWAS, we used sperm samples from stallions kept at the All-Russian Research Institute for Horse Breeding (ARRIHB, Ryazan Oblast), the Tersk Stud Farm No. 169 (Stavropol Krai), and the Perevozsky and Pochinkovsky studs (Nizhny Novgorod Oblast). Ninety-six animals (see Figure 1 for photographs of individual stallion examples) were sampled that represented the following 23 horse breeds: Akhal-Teke, Appaloosa, Arabian, Bashkir, Budyonny (or Budennovskaya), Don, French Trotter, German Warmblood, Hanoverian, Heavy Draft crossbreds, Holsteiner, Karabakh, Orlov Trotter, Rhenish German Coldblood (or Rhenish), Russian Heavy Draft, Russian Riding, Selle Français, Soviet Heavy Draft, Standardbred, Tersk, Thoroughbred, Trakehner (or Trakehnen), and Welsh Pony. There was an average of 4.2 males per breed, with the range of respective numbers for a single breed being 1 to 35 (Table 1). All stallions were healthy and varied in terms of sperm quality after thawing; therein, stallions with poorer quality were also included in the study.

Semen was collected at least three times from each stallion using an artificial vagina. Collection of semen samples, freezing and thawing were carried out by one same group of researchers in the spring-

summer period. A total of 288 semen samples, or three ejaculates from a stallion, were analyzed. There was an average of 12.5 samples per breed, the respective numbers per breed being ranged between 3 and 105. Where suitable for certain analyses, we combined samples from breeds with close breed characteristics into larger breed groups or removed few very small sized breeds to test if this could increase significance and accuracy of the obtained sperm parameters and comparisons.

**Semen examination**

Sperm was diluted at 1:3 (v/v) ratio with lactose-chelate-citrate-yolk (LCCY) medium containing 3.5% glycerin and frozen according to a standard technology (standard operating procedure) used at the ARRIHB and described in detail elsewhere [11,26–28]. Briefly, four-cornered aluminum tubes were used to package the diluted semen. After filling in a tube with 18 ml of diluted semen, the dimensions of the tube were as follows: length, 105 mm; width, 35 mm; and thickness, 4.5 mm. The frozen sperm concentration was 45–50 million/ml.

Fresh sperm progressive motility (PM) and post-thaw forward progressive motility (FPM) were measured in percentage of actively moving spermatozoa using a computer-assisted semen analysis (CASA [24]; Figure 2). The appropriate CASA system (ArgusSoft Ltd., St. Petersburg, Russia) and a Motic BA410 microscope (Motic, Hong Kong, China) were employed for this purpose. Comparison of mean values of motility traits was performed using the Student's *t*-test at a significance level of $p < 0.05$. Then, difference (DPM) between PM and FPM values was calculated as suggested elsewhere [29] and used for the subsequent GWAS analysis.

To estimate descriptive statistics, motility data processing was performed using Microsoft Excel. Differences in PM, FPM and DPM values between individual breeds and breed groups were evaluated for significance using the R software v. 4.1.0 [30], and the respective boxplots were produced using ggplot2 package [31].

Principal component analysis (PCA) plots and correlations were inferred from the sperm progressive motility data using R and libraries for the R environment [30]. Based on sperm motility data before

and after freezing, distribution of the studied 23 horse breeds was also tested with the web tools Phantasus [32] and ClustVis [33].

**SNP genotyping**

DNA was isolated from frozen semen samples using the phenol/chloroform method. DNA samples were genotyped using an Affymetrix high density chip, Axiom™ Equine Genotyping Array (Thermo Fisher Scientific, Waltham, MA USA). DNA samples with genotyping quality more than 98% at SNP loci were selected for further examination. SNP selection was carried out using the PLINK 1.9 software program [34] and minor allele frequency (MAF) > 0.05. After quality control, 306,522 variants were available for the further analysis.

**GWAS analysis**

GWAS were performed using EMMAX software [35] and an identity-by-state kinship matrix generated by EMMAX. Phenotype of each individual for the GWAS was determined by averaging the respective trait values from three samples per individual. To calculate effect of SNP on a trait, the following model was implemented:

$$Y = Xb + u + Br + e,$$

where $Y$ is a vector of phenotypes; $b$ is a SNP effect; $X$ is a design matrix of SNP genotypes; $u$ is a vector of additive genetic effects assumed to be normally distributed with the mean equal to 0 and (co)variance $\sigma^2 aG$, with $\sigma^2 a$ being additive genetic variance and $G$ being genomic relationship matrix; $Br$ is a breed effect; and $e$ is a vector of random residual effects.

Significance and suggestive levels for a SNP effect were set as $1.631204 \times 10^{-7}$ (0.05/306,522) and $3.262409 \times 10^{-6}$ (1.00/306,522), respectively. The genome-wide significance was assessed using the simpleM method in R, and calculation of effective number of independent tests was performed using $M_{eff}$ [36].

Based on the GWAS results and using the qqman package within the R software [37], a Manhattan and quantile-quantile (Q-Q) plot graphs were produced. Genes that coinciding with a candidate SNP genomic region or being close to it were determined using the *Equus caballus* (ECA; horse) genome assembly EquCab3.0 [38]. SNP information for relevant genes was retrieved using NCBI and Ensembl genomic browsers.

## RESULTS AND DISCUSSION

### Sperm motility differences

In this study, we additionally included in the analysis stallions with poor sperm cryotolerance, but since they were from different breeds, we expected to identify loci that included markers that did not depend on breed affiliation. For this, an additional adjustment was made in the analysis of GWAS for the breed effect.

As a result of assessing the quality of semen, the 288 stallion samples were analyzed in triplicate. The same ejaculates were investigated before and after freezing. Using CASA (Figure 2), we examined sperm progressive motility by individual stallion samples, breeds, and their groups. The results obtained by breed are shown in Table 1.

There were no significant differences in the produced values of sperm motility parameters in the stallion semen samples before and after freezing between either individual breeds (Table 1) or their groups (as represented with the respective boxplots shown in Figure 3).

PCA and clustering plots using the sperm progressive motility data did not reveal meaningful sample/breed clustering patterns (Figures 4A,C and Supplementary Figure S1A,B), although there were high correlations between PC1 and three tested sperm motility factors, i.e., PM, FPM and DPM (Figure 4B).

Although some breeds tended to have lower cryotolerance (as estimated by DPM) in contrast to other breeds, on average, there was no significant difference in semen characteristics between the studied

194 horse breeds/groups that might be due to a reduced sampling size of the breeds used in the study. On
195 the other hand, there may be best sires (refiners) and worst sires within each breed, i.e., there was
196 individual variability in most cases (as also observed in other studies [2,3,10,39]) that exceeded the
197 differences between breeds.
198 Overall, breeds in horses, as in other animal species, are however unlikely to differ significantly
199 among themselves in semen quality. These traits are too important for the overall "vitality" of a breed
200 to be expected to deteriorate. At the same time, within a breed, the variability of these characters may
201 well be observed. Therefore, we suggest that in general, frozen-thawed stallion sperm motility traits
202 are maintained at the same, averaged level across the breeds, while within-breed variation can be
203 quite pronounced as can be seen in the Figure 3 boxplots.
204
205 **GWAS implications and candidate genes**
206 A search for genomic associations with DPM resulted in one significant SNP, rs1141327473 (*p*-value
207 = 1.96e-06), located in the intron of the *NME8* gene on chromosome ECA4 (Table 2 and Figure 5).
208 The protein encoded by this gene, also known as sperm-specific thioredoxin 2 (*SPTRX2*), is probably
209 required at the final stages of maturation of the sperm tail in the testis and epididymis, where extensive
210 disulfide binding of fibrous proteins occurs. Mutations in this gene are involved in primary ciliary
211 dyskinesia-6 in humans. The protein expression was found at all stages of sperm maturation in the
212 tail [40]. Altogether, this information suggests that the *NME8* gene is involved in sperm tail formation
213 and its function is crucial for maintaining sperm motility.
214 The second suggestive SNP was rs1149048772 (*p*-value = 3.60e-08, with frequency of the C allele
215 being 0.12) located on ECA6 (Table 2 and Figure 5). One of the candidate genes for DPM found near
216 this SNP was *OR2AP1* (olfactory receptor family 2 subfamily AP member 1), a member of the
217 olfactory receptor family related to G-protein-coupled receptors (GPCR). Another gene, *OR6C4*
218 (olfactory receptor family 6 subfamily C member 4), is responsible for recognition and mediated by
219 the G protein. Expression of various GPCRs on the plasma membrane of human spermatozoa

suggested their involvement in the regulation of sperm motility, capacitation, and acrosome reaction [41]. Mutations in the *OR2AP1* gene were also suggested to cause adenocarcinoma of the prostate [42].

In addition, we investigated genomic associations for PM and FPM. While no significant SNP association was revealed for PM, we discovered the maximum effects for two SNPs within a region on ECA2, though insignificant ones. These were rs68590468 ($p$ = 3.99e-06) and rs396809330 ($p$ = 3.50e-05) in the introns of the *PHACTR4* (phosphatase and actin regulator 4) gene, the MAF values being 0.40 (G allele) and 0.36 (C allele), respectively. We would speculate that *PHACTR4* might be a putative candidate gene for FPM, although this would require further investigation and confirmation. The *PHACTR4* gene encodes a protein that is a member of the phosphatase and actin regulator family. It is known that members of the PHACTR family inhibit the activity of protein phosphatase 1 (PP1) and interact with actin and PP1 [43]; many transcript variants of the *PHACTR4* gene have been found that encode different isoforms. PP1 plays an important role in the control of glycogen metabolism that is critical for maintaining sperm motility [44].

**SNP genotypes associated with sperm cryotolerance**

Eventually, the analysis of the semen cryotolerance parameters was carried out with regard to their association with the identified SNP genotypes in stallions (Table 3). As a result, significant differences in the DPM values were revealed. Animals with the CT and CC genotypes at the rs1141327473 locus demonstrated a better cryotolerance despite poorer PM values. Examination of the SNP rs1149048772 genotypes also showed that the best resistance to freezing was in individuals with the CC and CT genotypes.

We also determined the occurrence frequency of minor alleles at significant SNPs as shown in Table 4. As a result of comparing the data from Tables 1 and 4, it was found that at SNP rs114132747 the occurrence of the C allele in the breeds studied was associated with a better cryotolerance (DPM).

Our study suggests that cryotolerance of stallion sperm is not a breed-dependent trait, and its association with polymorphic variants in the markers (genes) affecting sperm resistance to freezing should be considered intraspecific. The prevalence of cryotolerance genotypes can be slightly shifted towards an increase in the occurrence frequency only in those breeds in which artificial insemination with cryopreserved sperm is practiced in breeding. However, this practice is not common, and for some breeds it is absolutely impossible. These breeds include all purebred breeds (e.g., Arabian, Akhal-Teke, and Thoroughbred), in which the use of artificial insemination is prohibited. In breeding farms and large breeding companies, natural mating is preferred. Thus, the accumulation of polymorphisms associated with sperm cryotolerance in horse populations is minimized due to the intensive use of artificial insemination during reproduction. Therefore, the distribution of genotypes associated with cryotolerance of stallion sperm is not under selection pressure and seems natural.

Because of these observations, we believe that it seems reasonable to consider the association of the studied SNPs with indicators of sperm resistance to cryopreservation for the sample of breeds as a whole, and not for a single breed. In particular, when analyzing the whole sample of breeds (Table 4), a significant difference in DPM ($p < 0.01$) was identified between the CC, CT and TT genotypes for rs1149048772. The difference in motility (DPM) between native and cryopreserved sperm associated with this SNP was lower in the CC genotyped animals (13.33 ±4.98%; the motility index decreased by half) than that in the TT genotyped stallions (40.11 ±1.08%; the motility declined three times from the initial one). In stallions heterozygous for the rs1149048772 substitution, i.e., with the CT genotype, the decrease in motility was 30.38 ± 2.17%, which is less than half the PM. A significantly higher percentage of reduced sperm motility in the TT and CT stallions after cryopreservation indicates an effect of substitution of nucleotide C for T at the studied locus on DPM. The most favorable genotype for sperm cryotolerance at SNP rs1149048772 seemed to be the homozygous CC genotype. However, stallions with the CC genotype had a significantly reduced native sperm motility ($p < 0.01$). Specifically, PM of the CC stallions was significantly lower and amounted to 26.66 ±11.66% vs 51.75 ±5.16% in the CT males and 61.92 ±1.76% in the TT animals,

that is, more than twice between the two homozygous genotypes. This effect can be considered as a compensating effect between sperm quality and its survival (since exposure to ultra-low temperatures can be a stress factor for reproductive cells), requiring further investigation.

A similar effect was observed for SNP rs1141327473 C>T in the *NME8* intron. Particularly, PM in the stallions with the CC genotype was only 43.71 ± 4.21% vs the CT and TT stallions, in which motility was 60.08 ± 2.64 and 65.42 ± 2.39%, respectively ($p < 0.01$). The reduction in FPM, on the contrary, was the lowest in the stallions with the CC genotype (27.00 ± 2.42%) and higher in the CT stallions (37.61 ± 1.56%) and TT animals (42.95 ± 1.44%) at $p < 0.01$.

Thus, CC genotypes at the two SNPs studied could be linked to individuals with a higher sperm cryotolerance, but negatively affected the main quality indicator, i.e., progressive motility of native sperm.

SNPs detected by GWAS analyses are markers that do not always play a direct functional and/or regulatory role. They may be part of haplotypes, polymorphic variants of which can affect the level of expression or transcript isoforms. Many authors have shown the importance of polymorphisms in the gene promoters, intronic and other non-coding regions (e.g., [45–48]).

Recently, other authors found a region on ECA6 (at a distance of about 4.4Mb from that discovered by us) that was associated with the progressive motility of spermatozoa after thawing in stallions [49]. It contained the best-associated SNP in an intron within the *SCN8A* gene. The same study also revealed a suggestive SNP in an intergenic region near the *NOVA1* gene on ECA1.

In conclusion, we emphasize that for the effective reproduction in horse breeding it is important to know functional genes and genomic variants affecting stallion fertility and semen quality during cryopreservation [10,16–22]. The suggestive SNPs we detected here by GWAS can be relevant to the candidate genes *NME8*, *OR2AP1*, *OR6C4* and, possibly, *PHACTR4* that can be associated with sperm motility in males.

The detected horse candidate genes may functionally trot out effects of homologous genes in humans and other animals. The SNP markers and candidate genes we identified here for cryotolerance in

297 sperm as well as the respective genome regions can be helpful in studying the biological processes
298 underlying the formation and functioning of the reproductive system of stallions. Polymorphism in
299 the found candidate genes can be involved in sperm motility, suggesting their further detailed
300 investigation and potential use in horse reproduction and breeding programs.
301
302 **CONFLICT OF INTEREST**
303 The authors declare that they have no competing interests with regard to the reported research. We
304 also certify that there is no conflict of interest with any financial organization regarding the material
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306
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314
315 **SUPPLEMENTARY MATERIAL**
316 Supplementary file is available from:
317
318 Supplementary Figure S1. Distribution of the studied 23 horse breeds as built in the web tools
319 Phantasus ([32]; A) and ClustVis ([33]; B) using sperm motility data before and after freezing.
320
321 **REFERENCES**
322 1. Graham JK. Cryopreservation of stallion spermatozoa. Vet Clin North Am Equine Pract 1996;12:131-47.
323 https://doi.org/10.1016/s0749-0739(17)30300-0

2. Loomis PR, Graham JK. Commercial semen freezing: individual male variation in cryosurvival and the response of stallion sperm to customized freezing protocols. Anim Reprod Sci 2008;105(1-2):119-28. https://doi.org/10.1016/j.anireprosci.2007.11.010
3. Hoffmann N, Oldenhof H, Morandini C, Rohn K, Sieme H. Optimal concentrations of cryoprotective agents for semen from stallions that are classified 'good' or 'poor' for freezing. Anim Reprod Sci 2011;125(1-4):112-8. https://doi.org/10.1016/j.anireprosci.2011.03.001
4. Fiser PS, Ainsworth L, Fairfull RW. Evaluation of a new diluent and different processing procedures for cryopreservation of ram semen. Theriogenology 1987;28:599-607. https://doi.org/10.1016/0093-691x(87)90276-7
5. Sakhatsky NI, Tereshchenko AV, Artemenko AB. [An express-method of estimation of fertilizing ability of freezing-thawing of poultry spermatozoa]. Sel'skokhozyaistvennaya Biol [Agric Biol] 1987;22(12):77-80.
6. Luvoni GC, Colombo M. Cold case: Small animal gametes cryobanking. Theriogenology 2020;150:445-51. https://doi.org/10.1016/j.theriogenology.2020.02.047
7. Mel'nyk YuF, Mykytyuk DM, Bilous OV, et al. [Program of preservation of the gene pool of main types of farm animals in Ukraine for the period till 2015]. Kyiv, Ukraine: Aristey; 2009.
8. Weigend S, Romanov MN, Rath D. Methodologies to identify, evaluate and conserve poultry genetic resources. In: XXII World's Poultry Congress & Exhibition: Participant List & Full Text CD + Book of Abstracts; 2004 Jun 8-13: Istanbul, Turkey. Istanbul, Turkey: WPSA – Turkish Branch; 2004, p. 84.
9. Tagirov M, Artemenko A, Tereshchenko A. [Preservation of the poultry gene pool by cryoconservation]. Agrarnoe reshenie [Agrarian Solution] 2010;No. 10.
10. Gottschalk M, Metzger J, Martinsson G, Sieme H, Distl O. Genome-wide association study for semen quality traits in German Warmblood stallions. Anim Reprod Sci 2016;171:81-6. https://doi.org/10.1016/j.anireprosci.2016.06.002
11. Atroshchenko MM, Arkhangelskaya E, Isaev DA, et al. Reproductive characteristics of thawed stallion sperm. Animals 2019;9:1099. https://doi.org/10.3390/ani9121099
12. Kudinov AA, Dementieva N, Nikitkina E, Atroshchenko M, Musidrai A. 448 Late-breaking: GWAS analysis show QTL in horses which are characterized by sperm resistance to freezing. J Anim Sci 2019;97(Suppl 3):119-20. https://doi.org/10.1093/jas/skz258.247

13. Greaves IK, Rens W, Ferguson-Smith MA, Griffin D, Marshall Graves JA. Conservation of chromosome arrangement and position of the X in mammalian sperm suggests functional significance. Chromosome Res 2003;11:503-12. https://doi.org/10.1023/a:1024982929452
14. Foster HA, Abeydeera LR, Griffin DK, Bridger JM. Non-random chromosome positioning in mammalian sperm nuclei, with migration of the sex chromosomes during late spermatogenesis. J Cell Sci 2005;118(Pt 9):1811-20. https://doi.org/10.1242/jcs.02301
15. Sadraie M, Fowler KE, O'Connor RE, Griffin DK. Evaluation of aneuploidy of autosome chromosomes in boar sperm samples. Chromosome Res 2015;23:384-5. https://doi.org/10.1007/s10577-014-9447-3
16. Raudsepp T., McCue M.E., Das P.J., et al. Genome-wide association study implicates testis-sperm specific *FKBP6* as a susceptibility locus for impaired acrosome reaction in stallions. PLoS Genet 2012;8:e1003139. https://doi.org/10.1371/journal.pgen.1003139
17. Schrimpf R, Metzger J, Martinsson G, Sieme H, Distl O. Implication of *FKBP6* for male fertility in horses. Reprod Domest Anim 2015;50:195-9. https://doi.org/10.1111/rda.12467
18. Schrimpf R, Dierks C, Martinsson G, Sieme H, Distl O. Genome-wide association study identifies phospholipase C zeta 1 (PLCz1) as a stallion fertility locus in Hanoverian warmblood horses. PLoS ONE 2014;9:e109675. https://doi.org/10.1371/journal.pone.0109675
19. Usuga A, Rojano BA, Restrepo G. Association of the cysteine-rich secretory protein-3 (CRISP-3) and some of its polymorphisms with the quality of cryopreserved stallion semen. Reprod Fertil Dev 2018;30:563-9. https://doi.org/10.1071/RD17044
20. Restrepo G, Rojano B, Usuga A. Relationship of cysteine- rich secretory protein- 3 gene and protein with semen quality in stallions. Reprod Domest Anim 2019;54:39-45. https://doi.org/10.1111/rda.13309
21. Gottschalk M, Sieme H, Martinsson G, Distl O. Relationships among stallion fertility and semen traits using estimated breeding values of German Warmblood stallions. Theriogenology 2017;89:68-71. https://doi.org/10.1016/j.theriogenology.2016.10.011
22. Taylor JF, Schnabel RD, Sutovsky P. Identification of genomic variants causing sperm abnormalities and reduced male fertility. Anim Reprod Sci 2018;194:57-62. https://doi.org/10.1016/j.anireprosci.2018.02.007
23. Vasan SS. Semen analysis and sperm function tests: How much to test? Indian J Urol 2011;27:41-8. https://doi.org/10.4103/0970-1591.78424

24. Mortimer D, Mortimer ST. Computer-Aided Sperm Analysis (CASA) of sperm motility and hyperactivation. Methods Mol Biol 2013;927:77-87. https://doi.org/10.1007/978-1-62703-038-0_8
25. Suliman Y, Becker F, Wimmers K. Implication of transcriptome profiling of spermatozoa for stallion fertility. Reprod Fertil Dev 2018;30:1087-98. https://doi.org/10.1071/RD17188
26. Atroshchenko MM, Kalaschnikov VV, Bragina YeYe, Zaitsev AM. Comparative study of the structural integrity of spermatozoa in epididymal, ejaculated and cryopreserved semen of stallions. Sel'skokhozyaistvennaya Biol [Agric Biol] 2017;52:274-81. https://doi.org/10.15389/agrobiology.2017.2.274eng
27. Atroshchenko MM, Bragina EE, Zaitsev AM, Kalashnikov VV, Naumenkova VA, Kudlaeva AM, Nikitkina EV. Conservation of genetic resources in horse breeding and major structural damages of sperm during semen cryopreservation in stallions. Nat Conserv Res 2019;4(Suppl 2):78-82. https://doi.org/10.24189/ncr.2019.024
28. Nikitkina E, Musidray A, Krutikova A, Anipchenko P, Plemyashov K, Shiryaev G. Efficiency of tris-based extender Steridyl for semen cryopreservation in stallions. Animals 2020;10:1801. https://doi.org/10.3390/ani10101801
29. Dementieva NV, Kudinov AA, Pozovnikova MV, et al. Genome-wide association studies of cryostability of semen in roosters. Pol J Vet Sci 2020;23:461-3. https://doi.org/10.24425/pjvs.2020.134692.
30. R Core Team. R: A language and environment for statistical computing [Internet]. Vienna, Austria: R Foundation for Statistical Computing; 2021 [cited 2022 Jan 23]. Available from: https://www.R-project.org/
31. Wickham H. ggplot2: elegant graphics for data analysis. New York, NY, USA: Springer-Verlag; 2016.
32. Zenkova D, Kamenev V, Sablina R, Artyomov M, Sergushichev A. Phantasus: Visual and interactive gene expression analysis [Internet]. Bioconductor; 2018 [cited 2022 Jan 23]. Available from: https://doi.org/10.18129/B9.bioc.phantasus
33. Metsalu T, Vilo J. ClustVis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. Nucleic Acids Res 2015;43(W1):W566-70. https://doi.org/10.1093/nar/gkv468

34. Chang CC, Chow CC, Tellier LC, Vattikuti S, Purcell SM, Lee JJ. Second-generation PLINK: Rising to the challenge of larger and richer datasets. Gigascience 2015;4:s13742-015. https://doi.org/10.1186/s13742-015-0047-8

35. Kang HM, Sul JH, Service SK, et al. Variance component model to account for sample structure in genome-wide association studies. Nat Genet 2010;42:348-54. https://doi.org/10.1038/ng.548

36. Gao X. Multiple testing corrections for imputed SNPs. Genet Epidemiol. 2011;35(3):154–8.

37. Turner SD. qqman: an R package for visualizing GWAS results using Q-Q and manhattan plots. BioRxiv 2014;005165. https://doi.org/10.1101/005165

38. Kalbfleisch TS, Rice ES, DePriest MS Jr, et al. Improved reference genome for the domestic horse increases assembly contiguity and composition. Commun Biol 2018;1:197. https://doi.org/10.1038/s42003-018-0199-z

39. Aurich JE. Artificial insemination in horses—more than a century of practice and research. J Equine Vet Sci 2012; 32:458-63. https://doi.org/10.1016/j.jevs.2012.06.011

40. Miranda-Vizuete A, Tsang KYY, Jiménez A, et al. Cloning and developmental analysis of murid spermatid-specific thioredoxin-2 (SPTRX-2), a novel sperm fibrous sheath protein and autoantigen. J Biol Chem 2003;278:44874-85. https://doi.org/10.1074/jbc.M305475200

41. Urizar-Arenaza I, Osinalde N, Akimov V, et al. Phosphoproteomic and functional analyzes reveal sperm-specific protein changes downstream of kappa opioid receptor in human spermatozoa. Mol Cell Proteomics 2019;18(Suppl 1):S118-31. https://doi.org/10.1074/mcp.RA118.001133

42. Gerrin S, Sowalsky A, Balk SP, Ye H. Mutation profiling indicates high grade prostatic intraepithelial neoplasia as distant precursors of adjacent invasive prostatic adenocarcinoma. Prostate 2016;76:1227-36. https://doi.org/10.1002/pros.23212

43. Huet G, Rajakylä EK, Viita T, et al. Actin-regulated feedback loop based on Phactr4, PP1 and cofilin maintains the actin monomer pool. J Cell Sci 2013;126:497-507. https://doi.org/10.1242/jcs.113241

44. Fardilha M, Esteves SL, Korrodi-Gregório L, Pelech S, da Cruz E Silva OA, da Cruz E Silva E. Protein phosphatase 1 complexes modulate sperm motility and present novel targets for male infertility. Mol Hum Reprod 2011;17:466-77. https://doi.org/10.1093/molehr/gar004

45. Brinke I, Große-Brinkhaus C, Roth K, Pröll-Cornelissen MJ, Henne H, Schellander K, Tholen E. Genomic background and genetic relationships between boar taint and fertility traits in German Landrace and Large White. BMC Genet 2020;21:61. https://doi.org/10.1186/s12863-020-00865-z

46. Kosinska-Selbi B, Mielczarek M, Szyda J. Review: Long non-coding RNA in livestock. Animal 2020;14:2003-13. https://doi.org/10.1017/S1751731120000841

47. Serrano M, Ramón M, Calvo JH, Jiménez MÁ, Freire F, Vázquez JM, Arranz JJ. Genome-wide association studies for sperm traits in Assaf sheep breed. Animal 2021;15:100065. https://doi.org/10.1016/j.animal.2020.100065

48. Kumari A, Sedehizadeh S, Brook JD, Kozlowski P, Wojciechowska M. Differential fates of introns in gene expression due to global alternative splicing. Hum Genet 2022;141:31-47. https://doi.org/10.1007/s00439-021-02409-6

49. Gmel AI, Burger D, Neuditschko M. A novel QTL and a candidate gene are associated with the progressive motility of Franches-Montagnes stallion spermatozoa after thaw. Genes 2021;12:1501. https://doi.org/10.3390/genes12101501

451 **Table 1.** Motility parameters in stallion semen samples before and after freezing in the 23 horse
452 breeds studied

| Breeds | No. of males | PM, % | FPM, % | DPM, % |
|---|---|---|---|---|
| Akhal-Teke | 2 | 48.5±3.5 | 32.2±16.4 | 16.3±12.9 |
| Appaloosa | 1 | 43.0 | 26.0 | 17.0 |
| Arabian | 35 | 62.9±2.4 | 33.4±2.9 | 29.5±2.1 |
| Bashkir | 1 | 35.0 | 10.6 | 24.4 |
| Budyonny | 2 | 55.5±33.5 | 36.2±34.6 | 19.3±1.1 |
| Don | 2 | 31.0±1.0 | 13.5±1.5 | 17.5±2.5 |
| French Trotter | 7 | 77.1±5.0 | 44.3±6.7 | 32.8±3.1 |
| German Warmblood | 2 | 52.5±9.5 | 25.0±13.0 | 27.5±3.5 |
| Hanoverian | 1 | 59.2±9.6 | 32.3±8.0 | 26.9±3.4 |
| Heavy Draft crossbreds | 3 | 58.5±6.5 | 16.5±3.5 | 42.0±3.0 |
| Holsteiner | 3 | 59±19.5 | 30.0±13.0 | 29±6.5 |
| Karabakh | 1 | 42.0 | 20.3 | 21.7 |
| Orlov Trotter | 4 | 62.2±3.6 | 33.8±4.2 | 28.4±2.4 |
| Rhenish German Coldblood | 1 | 49.0 | 38.3 | 10.7 |
| Russian Heavy Draft | 1 | 10.0 | 5.8 | 4.2 |
| Russian Riding | 1 | 40 | 10 | 30 |
| Selle Fran çais | 1 | 61.0 | 46.0 | 15.0 |
| Soviet Heavy Draft | 11 | 58.1±4.0 | 30.0±5.2 | 28.1±2.5 |
| Standardbred | 6 | 63.7±4.4 | 36.1±4.1 | 27.6±3.7 |
| Tersk | 1 | 75.0 | 44.6 | 30.4 |

| | | | | |
|---|---|---|---|---|
| Thoroughbred | 1 | 35.0 | 13.7 | 21.3 |
| Trakehner | 6 | 67.5±2.3 | 42.9±2.5 | 24.6±3.3 |
| Welsh Pony | 3 | 70.7±12.9 | 36.3±10.5 | 34.4±5.3 |

453 PM, progressive motility; FPM, post-thaw forward progressive motility; DPM, difference between PM and FPM values

454

455 **Table 2.** Single nucleotide polymorphisms (SNPs) associated with difference between semen motility
456 before and after thawing, their minor allele frequency (MAF), and respective candidate genes

| SNPs | Chromosome | SNP position | *p*-value | Motif | MAF (allele) | Location | Candidate genes |
|---|---|---|---|---|---|---|---|
| rs1141327473 | ECA4 | 8415448 | 1.96E-06 | T/C | 0.48 (C) | intron | *NME8* |
| rs1149048772 | ECA6 | 74273805 | 3.60E-08 | T/C | 0.12 (C) | intergenic region | *OR2AP1*, *OR6C4* |

457

458 **Table 3.** Motility indices of native sperm and assessment of the effect of cryopreservation on

459 semen of stallions of various SNP genotypes

| Stallion SNP genotypes | No. of males | PM, % | FPM, % | DPM, % |
|---|---|---|---|---|
| rs1149048772 – CC | 3 | 26.66 ±11.66 [d] | 13.33 ± 6.69 | 13.33 ± 4.98 [a] |
| rs1149048772 – CT | 19 | 51.75 ± 5.16 | 21.38 ± 3.88 | 30.38 ± 2.17 [b] |
| rs1149048772 – TT | 74 | 61.92 ± 1.76 [e] | 21.80 ± 1.01 | 40.11 ± 1.08 [c] |
| rs1141327473 – CC | 18 | 43.71 ± 4.21 [f] | 16.71 ± 2.45 [l] | 27.00 ± 2.42 [i] |
| rs1141327473 – CT | 45 | 60.08 ± 2.64 [g] | 22.47 ± 1.65 [m] | 37.61 ± 1.56 [j] |
| rs1141327473 – TT | 33 | 65.42 ± 2.39 [h] | 22.47 ± 1.46 [n] | 42.95 ± 1.44 [k] |

460 Significant differences: [a-b, a-c, b-c, d-e, f-g, f-h, g-h, l-n, l-m, i-j, i-k, j-k] $p < 0.01$

461

462 **Table 4.** Frequency of minor alleles (MAF) in significant SNPs by breed

| Breed | No. of males | MAF (C) in SNPs | |
|---|---|---|---|
| | | rs1149048772 | rs1141327473 |
| Akhal-Teke | 2 | 0 | 0.75 |
| Appaloosa | 1 | 0 | 0.5 |
| Arabian | 35 | 0.1 | 0.39 |
| Bashkir | 1 | 0.5 | 0.5 |
| Budyonny | 2 | 0.25 | 0.5 |
| Don | 2 | 0.5 | 0.5 |
| French Trotter | 7 | 0.07 | 0.28 |
| German Warmblood | 2 | 0.25 | 0.25 |
| Hanoverian | 1 | 0 | 0 |
| Heavy Draft crossbreds | 3 | 0.33 | 1 |
| Holsteiner | 3 | 0.17 | 0.33 |
| Karabakh | 1 | 0.5 | 1 |
| Orlov Trotter | 4 | 0 | 0.5 |
| Rhenish German Coldblood | 1 | 0 | 0.5 |
| Russian Heavy Draft | 1 | 1 | 1 |
| Russian Riding | 1 | 0 | 0.5 |
| Selle Fran çais | 1 | 1 | 0.5 |
| Soviet Heavy Draft | 11 | 0.05 | 0.45 |
| Standardbred | 6 | 0.08 | 0 |
| Tersk | 1 | 0 | 0 |

| | | | |
|---|---|---|---|
| Thoroughbred | 1 | 0 | 1 |
| Trakehner | 6 | 0.17 | 0.25 |
| Welsh Pony | 3 | 0 | 0.83 |

463


**Figure 1.** Examples of individual stallions used in the present genome-wide association study for frozen-thawed sperm motility: (A) Khitmos, of the Trakehner breed, dark bay color; (B) Santrek, of the Trakehner breed, bay color; (C) Logotip, of the Orlov Trotter, black color; (D) Vympel, of the Orlov Trotter, black color; and (E) Zhasmin, of the Arabian breed, white color.

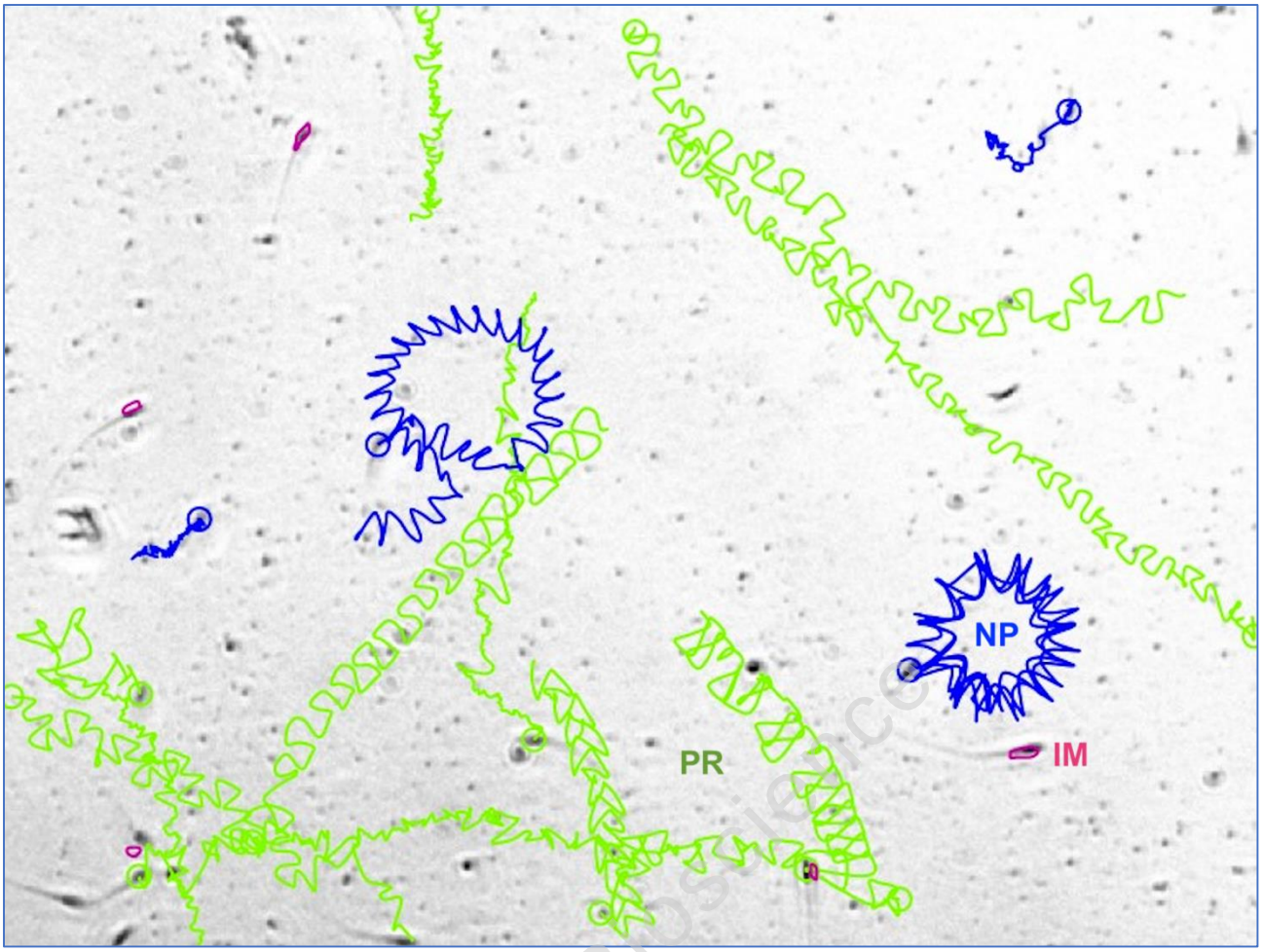
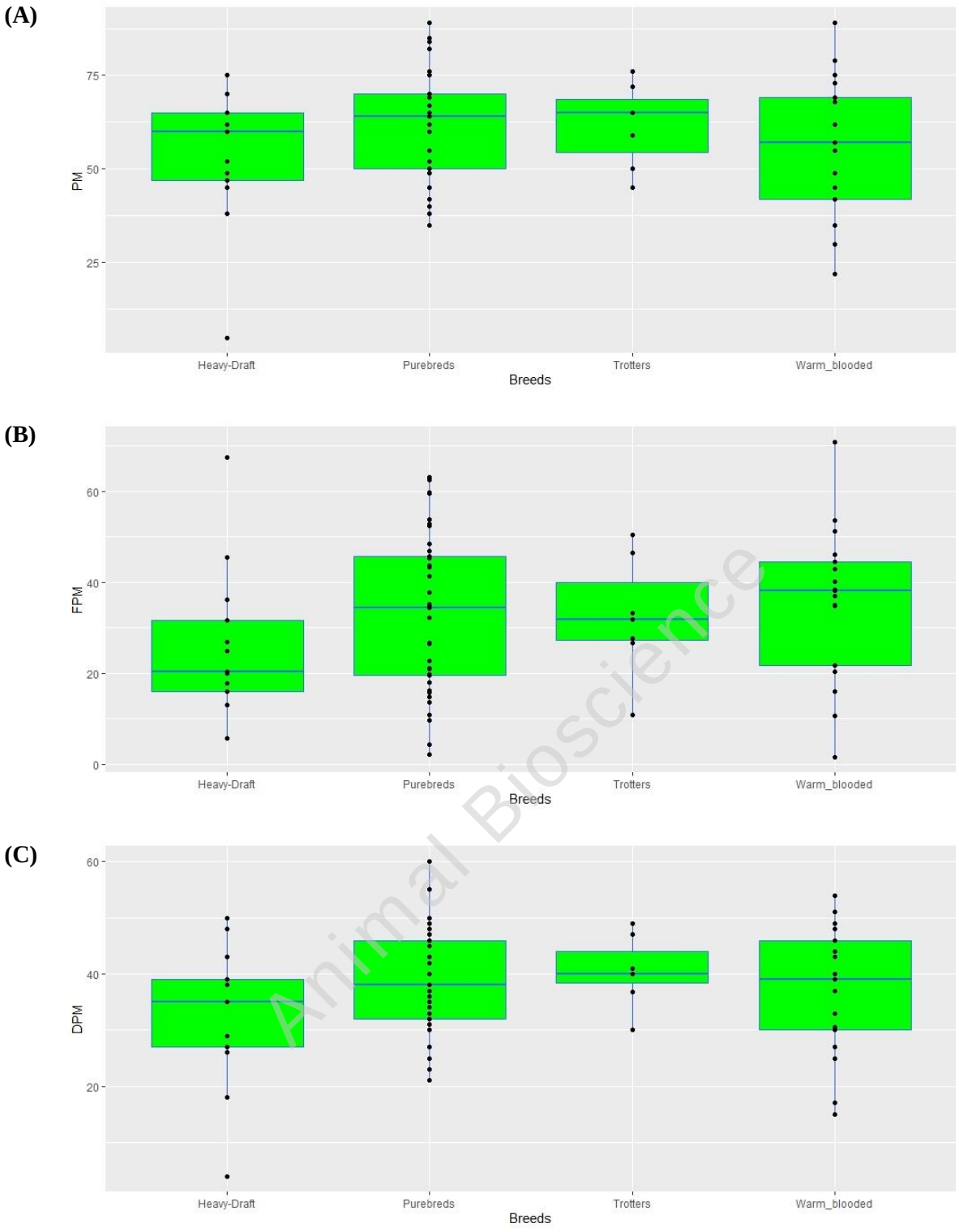


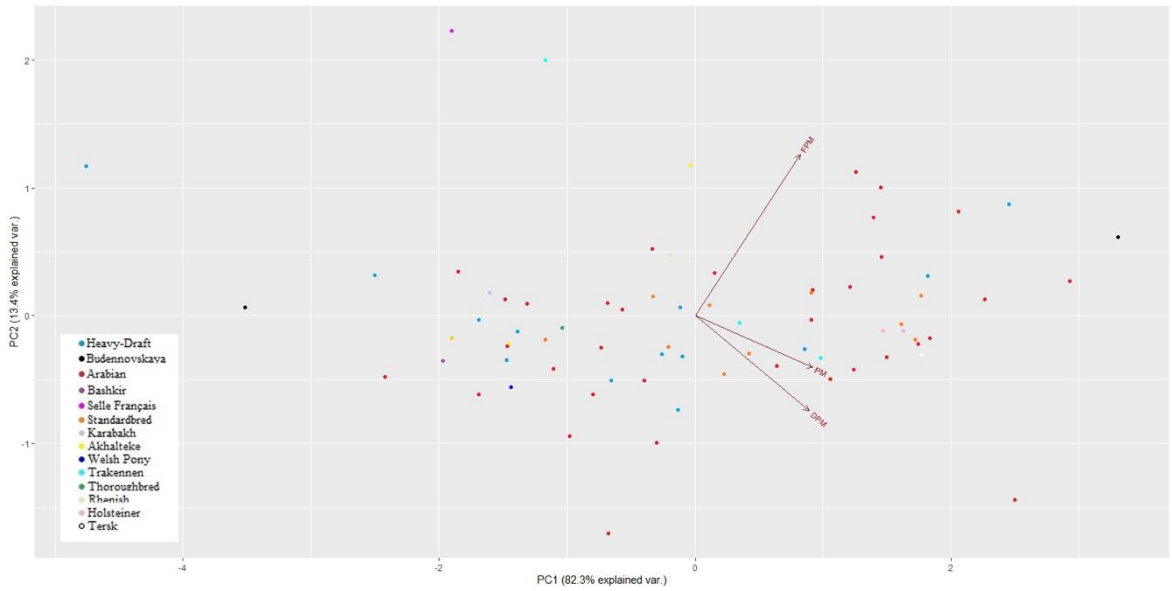

**Figure 2.** Example of the computer-assisted semen analysis (CASA) using a stallion semen sample. Sperm tracks generated by CASA: PR, progressive (green) tracks of individual sperms; NP, non-progressive (blue) tracks; and IM, immotile (or dead) sperm (red dots).



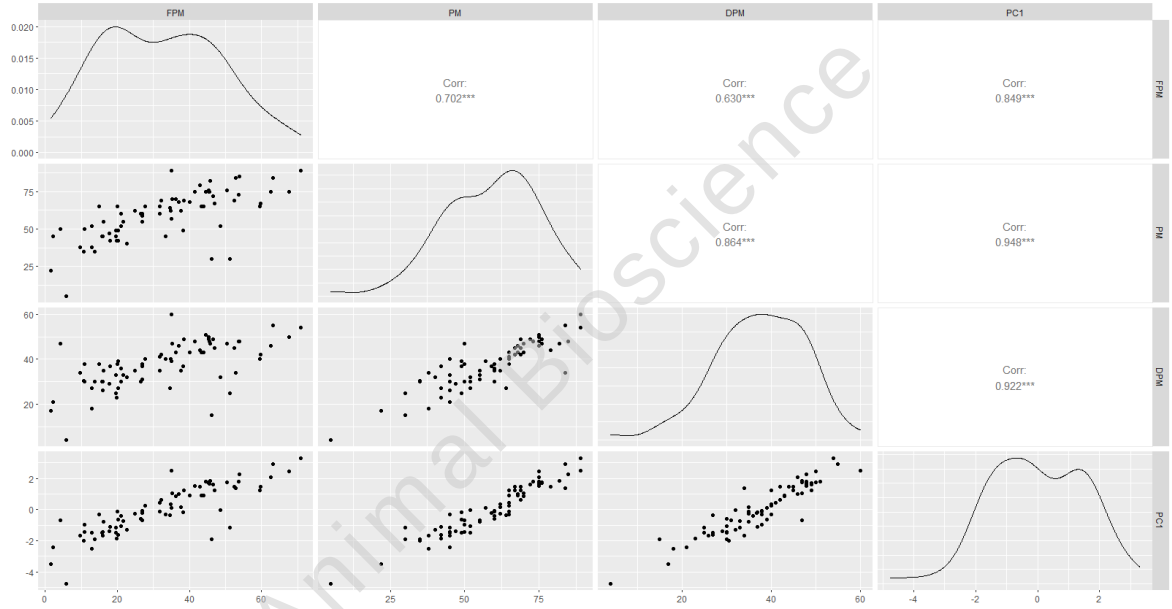



475 **Figure 3.** Quality parameters in stallion semen samples before and after freezing: (A) PM, fresh sperm
476 progressive motility; (B) FPM, post-thaw forward progressive motility; and (C) DPM, difference between
477 PM and FPM values. Breeds grouped: Heavy draft – Russian Heavy Draft, Soviet Heavy Draft; Purebreds –
478 Akhal-Teke, Arabian, Bashkir, Budyonny, Hanoverian, Karabakh, Rhenish German Coldblood, Tersk, and
479 Thoroughbred; Trotters – French Trotter, and Standardbred; and Warmblooded – Holsteiner, Orlov Trotter,
480 Russian Riding, Trakehner, and Welsh Pony.

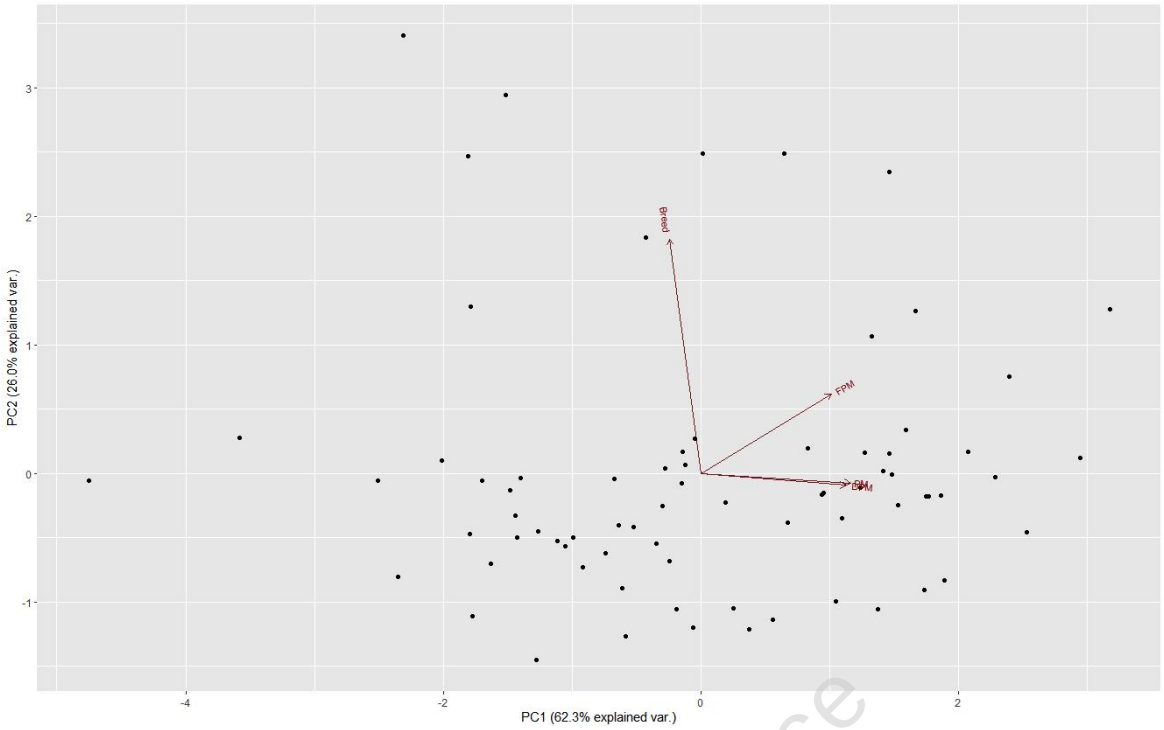
**(A)**




**(B)**



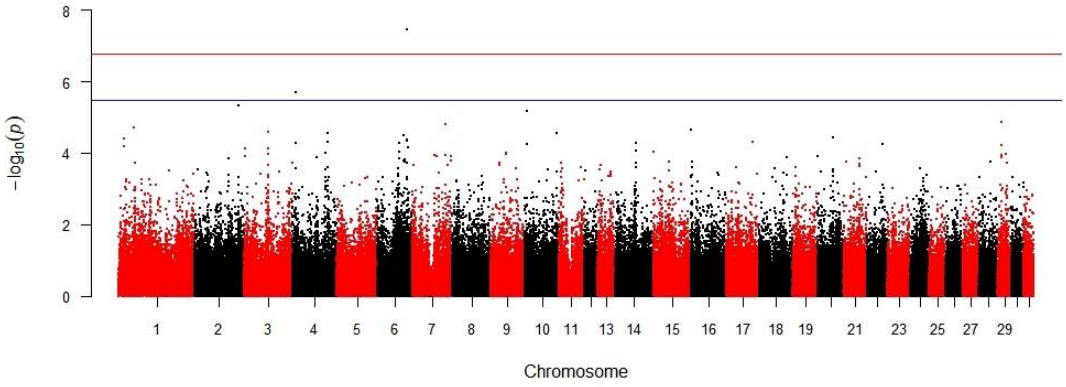
**(C)**



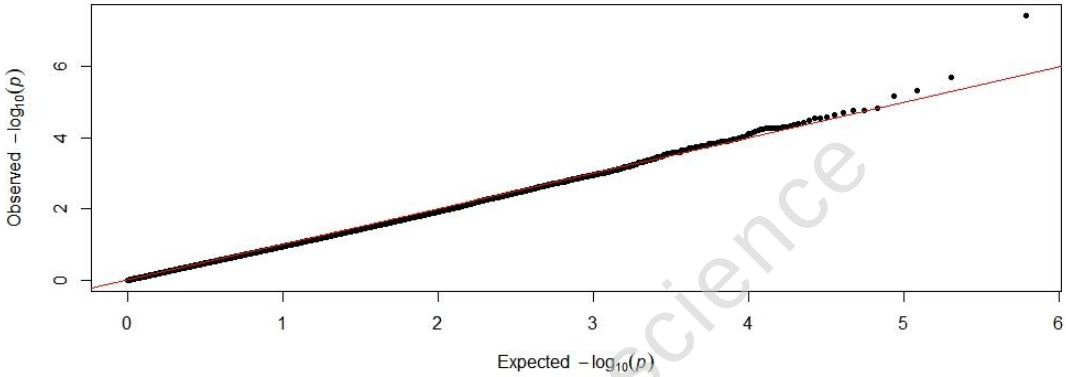

481 **Figure 4.** PCA plots based on the sperm progressive motility data (A and C) and correlations between PC1
482 and tested factors (B). For (A) and (B), three factors were used: progressive motility (PM), post-thaw forward
483 progressive motility (FPM), and difference (DPM) between PM and FPM values. For (C), the breed factor was
484 also tested as the fourth eigenvector. Breeds analyzed: Akhal-Teke, Arabian, Bashkir, Budyonny, Holsteiner,
485 Karabakh, Rhenish, Selle Fran çais, Standardbred, Tersk, Thoroughbred, Trakehner, Welsh Pony; and breed
486 group: Heavy draft (Russian Heavy Draft + Soviet Heavy Draft).

487

**(A)**




**(B)**






488 **Figure 5.** Manhattan (A) and quantile-quantile (Q-Q; B) plots resulted from the genome-wide association
489 study for sperm DPM. Blue line corresponds to the threshold of chromosome-wide suggestive levels for a
490 SNP effect ($p$ < 3.262409e-6), and red line represents the threshold of genome-wide significance ($p$ <
491 1.631204e-7). The Q-Q plot (B) shows the observed $p$-value plotted against the expected one.