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Original Research Article

Protamine 2 and phospholipase C zeta 1 are possible biomarkers for the diagnosis of male subfertility in frozen-thawed stallion semen

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ABSTRACT

Subfertility is one of the main issues in horse breeding and the study of mRNAs in sperm might help in elucidating the reasons that lead to this diagnosis. The present study aims at assessing the differences in the expression of 10 potential candidate genes in stallions of different fertility. Frozen-thawed semen of 29 stallions was included. Each sample was classified into two groups according to pregnancy rates (PR) achieved with this semen: "good fertility" (GF; n = 17; PR ≥ 30 %) or "poor fertility" (PF; n = 12; PR <20 %). All stallions underwent a breeding soundness examination (BSE) before semen production and were only included into the semen cryopreservation program when raw semen characteristics at BSE met minimal requirements. Semen was cryopreserved following European Union regulations and all stallions met the respective health requirements. Each sample was assessed for concentration (NucleoCounter SP-100), motility (CASA), membrane functionality (SYBR-14/PI), mitochondrial membrane potential (JC-1), morphology (SpermacStain), acrosome integrity (SpermacStain), membrane integrity (HOS test) and chromatin integrity (Aniline blue). Sperm RNAs were extracted using the Direct-zol RNA Miniprep Kit (Zymo Research) and RT-qPCR was performed for each target gene. ACTB and RPL32 were included as reference genes (RGs) for normalization. For each variable of each group, mean, standard deviation and SEM were calculated. The difference in gene expression levels between the GF and PF group were analyzed using the Mann-Whitney *U* test and Spearman's rank correlation. Significant results were considered with $p < 0.05$. Sperm quality parameters did not differ significantly between the two groups except for concentration, that was significantly higher in GF ($p = 0.043$). In GF a positive correlation was identified for *PRM1/PRM2* with $r = +0.6$, while *PRM1/ACR* ($r = -0.495$), *PRM2/ZPBP* ($r = -0.645$) and *CRISP3/ACR* ($r = -0.551$) were inversely correlated. In PF direct correlations were registered for *PRM1/PRM2* ($r = +0.629$), *PRM1/PRM3* ($r = +0.657$), *PRM2/SPA17* ($r = +0.685$), *SPA17/PLCZ1* ($r = +0.786$) and *PRM3/ACR* ($r = +0.627$). In the total sample (GF + PF), positive correlations were detected for *PRM1/PRM2* ($r = +0.625$), *PRM1/PRM3* ($r = +0.368$); *PRM2/SPA17* ($r = +0.465$), *SPA17/PLCZ1* ($r = +0.637$) and *PLCZ1/ZAN* ($r = +0.587$). Only two of the genes considered were differentially expressed in the 2 groups: *PRM2* and *PLCZ1*, that were significantly ($p < 0.05$) overexpressed in the GF group. Stallions frozen-thawed semen with higher expression levels of *PRM2* and *PLCZ1* are more likely to belong to animals with a good pregnancy rate. Further studies are needed to investigate the role of sperm transcripts in male subfertility in stallions.

1. Introduction

According to the World Health Organization (WHO), one in eight of reproductive age couples worldwide are affected by subfertility [1]. Irrespective of species, male subfertility diagnosis still mainly focuses on the assessment of sperm characteristics like concentration, motility, and morphology, but in the last decade the study of transcriptomics and proteomics led to important developments in understanding the hidden mechanisms involved in fertility or infertility, respectively [2–4].

Determination of the expression of specific RNAs in spermatozoa may allow for the development of novel techniques for predicting fertility. Expression anomalies of RNAs play a role in idiopathic

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infertility that can reflect on the poor performance of livestock [5] because each step of the reproduction mechanism (from spermatogenesis to fertilization) is mediated by a number of proteins. Genes encoding these proteins may be considered as potential candidates for male fertility traits [6]. Identification of such biomarkers may eventually help to improve the reproductive efficiency of farm animals [7]. In human and mice, more than 200 genes and a large number of proteins involved in male reproduction and fertilization were identified, but only limited information is available for other species, including the horse [8,9].

In 2017, Parthipan et al. detected that increased expression of genes involved in apoptosis and homeostasis mechanisms, *apoptosis – related cysteine peptidase* (*CASP3*), *TNFRSF1A – associated via death domain* (*TRADD*), *ubiquitin – conjugating enzyme E2D3* (*UBE2D3*) and *heat shock transcription factor, Y linked 2* (*HSFY2*) is responsible for low conception rates (<40 %) in bulls [10]. In this species, although an involvement of six different genes, including *protamine 1* (*PRM1*), in the fertilization process was characterized, only the expression of *coiled coil domain containing 174* (*CCDC174*), which is involved in motility and acrosomal reaction, was identified to be of interest for fertility prediction [11].

In stallions, transcriptomics studies on sperm are still limited because of sperm species-specific attributes that require to continuously adjust the RNA extraction protocols [12]. Since 2007, more than 60 genes have been identified to be related with stallion fertility, including *phospholipase C zeta 1* (*PLCZ1*), *spermatogenic associated 1* (*SPATA1*), *angiotensin converting enzyme* (*ACE*), *sperm autoantigenic protein 17* (*SP17*) and *follicle stimulating hormone* (*FSHB*) [13–15].

Cryopreservation of stallion semen is still attracting increasing interest. Although stallions with good raw semen quality have in average a better chance to produce frozen semen with acceptable quality [16], but this does not guarantee a good fertility of their frozen-thawed semen [17]. The identification of markers for the prediction of frozen-thawed semen fertility would therefore be highly beneficial and avoid disappointment in stallion owners and breeders. The aim of the present study was to identify differences in the expression of selected genes in stallions where frozen semen was proven to be of either good or poor fertility although semen characteristics met minimal requirements and did not differ between groups. We hypothesized that at least some of the analyzed genes would be differently expressed in semen of the two groups and could thus be of interest for predicting fertility.

2. Materials and methods

2.1. Study design

Frozen–thawed semen from 29 healthy stallions was included into the study. Stallions were of different breeds (7 Arabian Horses, 1 Paint horse, 1 Quarter Horse, 16 Warmblood, 1 Andalusian, 1 Welsh COB, 1 Thoroughbred, and 1 Shetland pony), aged 3–21 years old and ranging in weight from 180 to 580 kg (body condition score 2,5-3,5). They were housed at the Centre for Artificial Insemination and Embryo Transfer of Vetmeduni Vienna in individual boxes with access to an external paddock for two or 3 h per day. They were kept at rest and fed hay and concentrates three times a day. Water was provided ad libitum. Data collected on analysis of frozen-thawed semen of 15 of these stallions were in part included into a previous publication by our group [18]. Semen had been produced for commercial purposes at the Centre for Artificial Insemination and Embryo Transfer of Vetmeduni Vienna that is approved for stallion semen production according to European Union regulations (Directive 65/92 EEC). Semen was included in the present study if it had been used for insemination of at least five different mares (age 3–15 years) with no previous history of reproductive anomalies. Semen analyzed in this study was always produced in the same time period as the semen used for insemination and if possible, was taken from the same batches as used for insemination. All mares underwent a breeding soundness examination including negative uterine culture and cytology before artificial insemination (AI). In accordance with WBFSH recommendations ("WBFSH, World Breeding Federation for Sport Horses: Semen standards"; accessed on 19 May 2020; available online: http://www.wbfsh.org/files/Semen%20standards.pdf), semen was classified acceptable for AI when progressive motility after thawing was ≥35 %. One insemination dose consisted of at least 250 × 10^6 progressively motile spermatozoa after thawing. Semen was classified retrospectively, i.e. according to the previously achieved pregnancy rate (PR) as having "good fertility" (GF; n = 17; PR ≥ 30 %) or "poor fertility" (PF; n = 12; PR <20 %). In the present investigation, straws from one semen batch of each of these stallions were included. Semen had been produced for regular commercial AI programs after stallions underwent a breeding soundness examination (BSE) where their raw semen characteristics met minimal requirements [16]. Semen was cryopreserved following European Union regulations (Directive 65/92 EEC) and all stallions met the respective health requirements. According to the Austrian law for the protection of experimental animals (Law Decree n. 26 issued on 4 March 2014, art. 2), the approval by an ethical committee for the experiment is not required under the circumstances that this trial was carried out. The owner's informed consent was always collected.

2.2. Semen collection and cryopreservation

Semen freezing was performed as previously described [16]. Briefly, ejaculates were collected on a breeding dummy using an artificial vagina (Hannover model; Minitube, Tiefenbach, Germany) fitted with an inlaying filter (Minitube) for removal of the gel fraction. Semen was diluted 1:1 (v:v) with EquiPlus extender (Minitube) centrifuged at 700×*g* for 12 min. The supernatant was removed, and the pellet was resuspended with Ghent freezing extender (Minitube) at a ratio of 1:1 (v:v). The semen was filled into 0.5 mL straws automatically sealed at room temperature (MPP Uno, Minitube). Straws were frozen using a computer-controlled rate freezer at 20 °C (IceCube 14 M; Sylab, Purkersdorf, Austria). Semen was first cooled to 5 °C at a cooling rate of 0.3 °C/min, then to −25 °C within 3 min (10 °C/min) and finally to −140 °C (25 °C/min). Straws were removed from the freezing chamber and immediately plunged into liquid nitrogen. After cryopreservation of each semen batch, at least one straw was used for the determination of post-thaw semen characteristics. The straw was thawed in a water bath at 37 °C for 15 s. The thawed semen was transferred into an Eppendorff tube and left at room temperature for at least 10 min before further analysis. Post-thaw quality of semen was evaluated for sperm concentration, total and progressive motility as well as sperm membrane integrity as described above. Semen was classified acceptable for insemination when progressive motility after thawing was ≥35 %. One insemination dose consisted of at least 250 × 10^6 progressively motile spermatozoa after thawing [16].

2.3. Analysis of sperm characteristics in frozen-thawed semen

Sperm concentration was measured using a NucleoCounter (ChemoMetec, Allerød, Denmark) as described [19]. Semen motility and sperm kinetic parameters were analyzed by computer–assisted sperm analysis (CASA; SpermVision, Minitube) as described [20,21] with the modification that analysis was performed on pre-warmed glass slides filled by placing a volume of 10 μL on the designated place, followed by immediate application of the coverslip (WHO prepared motility slide, [22]). The CASA characteristics total motility (TM, %), progressive motility (PM, %), average path velocity (VAP, μm/s), curvilinear velocity (VCL, μm/s), straight – line velocity (VSL, μm/s), distance average path (DAP, μm), distance curved line (DCL, μm), distance straight – line (DSL, μm), amplitude of the lateral head displacement (ALH, μm), straightness (STR, %), linearity (LIN, %), wobble coefficient (WOB, %) and beat – cross frequency (BCF, Hz) were considered. For each sample, 7 fields and a total of at least 700 sperm were analyzed, and the mean values were calculated by the CASA system.

Membrane integrity (MI) was assessed using SYBR14/PI fluorescent

stain (Minitube, Tiefenbach, Germany) as described [20]: 100 μL of semen was mixed with 2 μL of SYBR-14/PI and incubated for 10 min at room temperature (RT) in darkness. One droplet was placed onto a glass slide, covered with a glass coverslip, and evaluated by fluorescence microscopy at magnification ×40 (Olympus AX70; Olympus, Vienna, Austria; U-MWB filter block, BP420–480 excitation filter, BA515 suppressor filter, dichromatic mirror: DM500). Green spermatozoa were considered membrane-intact (alive), while dead spermatozoa were stained red. Spermatozoa were recognized and counted according to their color by the CASA system (15 fields).

In addition, the hypo – osmotic swelling (HOS) test was used for the evaluation of membrane functionality (MF): 10 μL of semen were mixed with 100 μL of fructose solution (100 mOsm) and incubated at 37 °C for 1 h. After incubation one droplet of the solution was placed onto a glass slide and analyzed by light microscopy at ×40 magnification. Sperm with coiled tails were considered HOS+ (functionally intact plasma membrane) [23].

Acrosome integrity and sperm morphology were evaluated using the Spermac Stain kit (Minitube) as described by Runcan et al. [24]. A drop of the sample was smeared onto a glass slide, left to dry at RT and then dipped into the fixative solution for 5 min. The slide was then washed in distilled water and left to dry at RT. Once dried it was stained for 3 min in stain A, 1 min in stain B and 30 s in stain C. After each staining passage, the slide was washed in distilled water. At the end of the staining procedure, the slide was left to dry at RT and observed with light microscopy at ×1000 magnification. Spermatozoa were considered to have an intact acrosome when the anterior acrosomal region stained green and the posterior post acrosomal region was red – pink. For morphology spermatozoa were classified as normal or with head, midpiece or tail anomalies.

Mitochondrial membrane potential (MMP) was assessed using JC – 1 fluorescence staining [25]: 150 μL of semen were incubated for 8 min in the dark with 3 μL of JC – 1 (1530 μM). After incubation a drop of the solution was put onto a glass slide, covered with a glass coverslip, and analyzed by fluorescence microscopy at ×40 magnification (ZEISS Observer Z1, Jena, Germany). Spermatozoa with bright orange midpiece were considered to have a high mitochondrial membrane potential.

Sperm chromatin condensation (SCC) was assessed with aniline blue staining as previously described [26]. Semen samples were centrifuged twice in PBS (1:1) for 10 min at 1500 rpm. The pellet was resuspended with 1 % SDS in sodium citrate and incubated for 15 min at 4 °C, then spread onto a glass slide, and allowed to dry. The smears were fixed in 3 % buffered glutaraldehyde in 0.2 mol/L phosphate-buffered saline (pH 7.2) for 20 min. The slides were finally stained with 5 % aqueous aniline blue mixed with 4 % acetic acid (pH 3.5) for 15 min at RT. The slides were assessed with brightfield microscopy at ×40 magnification (ZEISS Observer Z1). Spermatozoa with blue – stained head were considered to have abnormal chromatin condensation.

For each test, at least 100 cells were counted.

2.4. mRNA isolation and qRT–PCR from frozen-thawed semen

Density gradient centrifugation was performed prior to RNA extraction: 2500 μL of semen (5 straws) was layered on EquiPure™ (Nidacon, International AB, Mölndal, Sweden) and centrifuged at x 800 g for 20 min. Pellet was removed using an aspiration needle, resuspended in PBS to a volume of 500 μL, sperm concentration was determined (see 2.3), and the resuspended pellet centrifuged at 1500 rpm for 10 min. The supernatant was again removed, the pellet resuspended in PBS and another centrifugation performed as described. The pellet was resuspended in PBS to a final concentration $\geq 100 \times 10^6$ sperm/mL.

The cells were collected by centrifugation (x 1500 g for 4 min at 4 °C), resuspended in 2 mL somatic cell lysis buffer (0.05 % sodium dodecyl sulphate, 0.25 % Triton X-100; Sigma-Aldrich, St. Louis, MO, USA) and incubated on ice for 10 min. Afterwards, the cells were washed twice with PBS, then resuspended in 800 μL TRI Reagent (Zymo Research, Irvine, CA, USA) and transferred into lysis tubes pre-filled with 100 mg 0.2 mm stainless steel beads (Next Advance, Troy, NY, USA). Sample homogenization was performed on a MagNA Lyser instrument (Roche, Rotkreuz, Switzerland) at 7000 rpm for 30 s, conducting a total of 3 homogenization rounds. The samples were chilled on ice for 1 min after each homogenization. RNA extraction including an on-column DNase I treatment was done with the Direct-zol RNA Miniprep Kit (Zymo Research) according to the manufacturer's recommendations. The absence of ribosomal RNA from contaminating somatic cells was confirmed by capillary electrophoresis on the 2100 Bioanalyzer with the RNA 6000 Pico Kit (Agilent, Santa Clara, CA, USA). The iScript Explore One-Step RT and PreAmp Kit (Bio-Rad, Hercules, CA, USA) was used for an additional DNase digestion, reverse transcription, and cDNA pre-amplification of 100 ng RNA input according to the recommended protocol with 12 pre-amplification cycles. The amplified cDNA was diluted 1:10 for further analyses. RT-qPCR was done in 20 μL reaction volumes including 1x HOT FIREPol EvaGreen qPCR Mix Plus ROX (Solis BioDyne, Tartu, Estonia), 200 nM of each primer and 2 μL diluted cDNA. Primer sequences are listed in Supplemental Table 1 [27]. ACTB and RPL32 were included as reference genes (RGs) for normalization [28]. All samples were analyzed in duplicates on an AriaMx Real-Time PCR System (Agilent) with following temperature profile: 95 °C for 12 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min, followed by a melting curve step (60 °C–95 °C). The RefFinder tool was used to assess the RG stability [29].

2.5. Statistical analysis

Statistical analysis was performed with SPSS version 27.0 (SPSS-IBM, Armonck, NY, USA) and GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA). For each variable of each group, mean, standard deviation and SEM were calculated. Data were tested for normality and homogeneity of variances using the Kolmogorov–Smirnov and the Levene test, respectively. Differences in semen characteristics between groups GF and PF were analyzed by Mann-Whitney test. The sperm count before and after density gradient centrifugation was compared by General Linear Model ANOVA for repeated measures with group (good vs. poor fertility) as within subject factor. Sperm recovery (%) after density gradient centrifugation was calculated as by the formula: (number of sperm recovered after density gradient centrifugation/ number of sperm submitted to density gradient centrifugation) x100. For *qRT–PCR*, the mean of ACTB and RPL32 was used for normalization because both RGs were similarly stable expressed. Mean Cq values were corrected for PCR reaction efficiencies (Supplemental Table 2) and relative expression changes were calculated with the 2-ΔΔCT method. Differences between the GF and PF group were analyzed by Mann-Whitney *U* test. Spearman's rank correlation was used to assess the relationship among genes. A p value < 0.05 was considered statistically significant.

3. Results

Semen analysis revealed that the mean concentration of frozen-thawed semen was higher in GF than in PF stallions ($p < 0.05$). There were no differences between groups for all other sperm characteristics assessed (Tables 1 and 2). Density gradient centrifugation, however, decreased sperm recovery after density gradient centrifugation more in PF than in GF stallions (recovery rate GF 11.1 ± 1.4 %, PF 7.9 ± 1.1 %; treatment x group $p < 0.05$).

In *PLCz1* and *PRM2*, but in no other of the genes considered in this study, a greater ($p < 0.05$) mRNA abundance was determined in the frozen-thawed semen of stallions from the GF in comparison to the PF group (Fig. 1). There was also no difference in the ratio of mRNA abundance for *PRM2* to *PRM1* (data not shown). For the genes *ZAN*, *ZPBP*, *PLCZ1* and *ACR*, mRNA could not be detected in 13, 11, 7 and 1 out of the 29 samples, respectively. The mean Cq values corrected for

Table 1
Results for the semen concentration and motility characteristics of frozen-thawed stallion semen from ejaculates characterized being of good fertility (GF) or poor fertility (PF). Results are presented as mean ± SEM. TM: total motility; PM: progressive motility; DAP: distance average path; DCL: distance curved line; DSL: distance straight – line; VAP: average path velocity, VCL: curvilinear velocity, VSL: straight – line velocity, STR: straightness; LIN: linearity; WOB: wobble coefficient; ALH: amplitude of the lateral head displacement; BCF: beat – cross frequency. *P < 0.05.

	GF (n = 17)	PF (n = 12)	P - value
	Mean ± SEM	Mean ± SEM	
Concentration	346.36 ± 30.27	260.82 ± 59.71	0.043*
TM	69.8 ± 3.39	70.02 ± 3.38	0.711
PM	58.8 ± 3.85	60.01 ± 3.85	0.948
DAP	38.6 ± 1.21	35.77 ± 2.08	0.195
DCL	72.22 ± 2.29	67.20 ± 3.74	0.283
DSL	33.72 ± 1.02	31.47 ± 1.95	0.180
VAP	83.69 ± 2.78	77.80 ± 4.69	0.227
VCL	156.1 ± 5.12	145.55 ± 8.19	0.325
VSL	73.17 ± 2.35	68.52 ± 4.38	0.211
STR	0.87 ± 0.007	0.87 ± 0.008	0.983
LIN	0.46 ± 0.009	0.46 ± 0.01	0.879
WOB	0.53 ± 0.008	0.53 ± 0.01	0.948
ALH	3.46 ± 0.15	3.41 ± 0.13	0.948
BCF	33.37 ± 0.87	33.59 ± 0.58	0.948

Table 2
Results for semen characteristic analyzed by microscopic evaluation of frozen-thawed stallion semen from ejaculates characterized being of good or poor fertility. Mean ± SEM for membrane integrity (MI), membrane functionality (MF), mitochondrial membrane potential (MMP), acrosome integrity (ACR), morphology (normal; head anomalies; midpiece anomalies; tail anomalies) and sperm chromatin condensation (SCC) in frozen-thawed stallion semen. Parameters are divided by group in good fertility (GF) and poor fertility (PF). *P < 0.05.

	GF (n = 17)	PF (n = 12)	P - value
	Mean ± SEM	Mean ± SEM	
MI	55.16 ± 3.08	55.21 ± 2.82	0.777
MF	50.41 ± 2.13	49.88 ± 2.76	0.679
MMP	54.24 ± 3.25	50.54 ± 3.44	0.679
ACR	86.91 ± 0.90	86.88 ± 1.71	0.845
Normal	79.30 ± 1.72	78.83 ± 2.09	0.499
Head	6.35 ± 1.02	6.71 ± 1.54	0.948
Midpiece	3.88 ± 0.68	5.33 ± 0.90	0.245
Tail	9.82 ± 0.96	9.08 ± 1.54	0.679
SCC	91.32 ± 2.35	93.13 ± 1.16	0.879

PCR efficiency for each gene analyzed in the semen samples included into the present study are shown in Supplemental Table 2.

The results of the correlation analysis are presented in Fig. 2. In GF, a positive correlation was identified for *PRM1/PRM2* ($r = +0.6$; $p < 0.05$), while negative correlations were found for *PRM1/ACR* ($r = -0.495$; $p < 0.05$), *PRM2/ZPBP* ($r = -0.645$; $p < 0.05$) and *CRISP3/ACR* ($r = -0.551$; $p < 0.05$). In the group PF, only positive correlations were revealed for *PRM1/PRM2* ($r = +0.629$; $p < 0.05$), *PRM1/PRM3* ($r = +0.657$; $p < 0.05$), *PRM2/SPA17* ($r = +0.685$; $p < 0.05$), *SPA17/PLCZ1* ($r = +0.786$; $p < 0.05$) and *PRM3/ACR* ($r = +0.627$; $p < 0.05$). When considering all samples (GF + PF), positive correlations were detected for *PRM1/PRM2* ($r = +0.625$; $p < 0.01$), *PRM1/PRM3* ($r = +0.368$; $p < 0.05$); *PRM2/SPA17* ($r = +0.465$; $p < 0.05$), *SPA17/PLCZ1* ($r = +0.637$; $p < 0.01$) and *PLCZ1/ZAN* ($r = +0.587$; $p < 0.05$).

4. Discussion

In the context of fertility evaluation, the most complicated task is to obtain significant fertility data. This is particularly true for the horse because of the limited number of insemination doses that are produced and used and because of the limited information that, in most cases, is available with regard to mares, especially when dealing with field fertility data. For these reasons, the need of an objective protocol to assess semen fertility is always more impellent. In recent years, the study of semen transcripts has been a subject of increasing interest, starting from humans, and expanding into veterinary medicine. Sperm RNA extraction is, however, a challenging procedure that, due to species-specific features of sperm requires continuous adjustments [30]. The most important factor that affects RNA extraction is the percentage of retained histones (e.g., 1 % of retained histones in rat sperm vs. 15 % in human sperm chromatin) and the different amount of RNA in each individual sperm [31]. These features make RNAs more easily accessible in some species compared to others. To date, great progress in this regard was made in domestic animal species like cattle [32,33]. In the present investigation conducted in stallion semen, mRNA of the genes of interest was not always detectable in all samples. This applies for 4 out of the 11 candidate genes. If semen transcripts should be applied as biomarkers to predict fertility of a given sample or a given individual, the non-detectability of mRNAs is a major setback. Further improvement of the respective techniques is therefore required.

The aim of this study was to identify sperm transcripts that might help to predict fertility of frozen-thawed stallion semen. Therefore, animals were retrospectively assigned to two groups based on pregnancy rates achieved with frozen-thawed semen. Semen characteristics between groups did not differ with regard to sperm characteristics assessed by analysis in the semen laboratory. Considering that each semen batch was used for insemination because it met the minimum quality requirements mentioned above, the situation is comparable to the condition defined as “male factor infertility” in humans. This affects 35%–40 % of patients who produce semen with physiological characteristics but unable of oocyte activation after fertilization [34]. Similar to the situation in the present study, standard semen assessment is therefore not suitable to identify frozen semen with a low fertility potential. The study of sperm transcripts might be a valid tool to explain sub- or infertility in such cases.

In this context, the fact that frozen-thawed semen from the GF and PF groups differed with regard to sperm concentration is interesting and extends similar findings from a previous publication [18] to a larger population of stallions. The finding supports the assumption that a greater concentration of frozen-thawed semen is beneficial for its fertility. To the best of our knowledge, there is no apparent explanation for this finding. Higher sperm concentration may be advantageous to escape uterine mechanisms aiming at sperm elimination and thus ensuring that sufficient sperm arrive in the oviduct (reviewed by Maitan et al. [35]). It is interesting to note that more sperm were recovered from GF than from PF stallions after density gradient centrifugation that was primarily performed to remove somatic cells prior to RNA extraction. This is a further proof for differences in sperm characteristics between GF and PF stallions because density gradient centrifugation selects for sperm with better quality [36,37].

We decided to include *phospholipase C zeta 1* (*PLCZ1*) in the transcript analysis because it was previously identified as the most likely reason of low fertility in stallions and other species [15,38,39]. The protein is mainly expressed in the acrosomal and post acrosomal sperm region as well as the midpiece and principal piece. It is considered essential for spermatogenesis and oocyte activation through sperm induced Ca2+-oscillation that promotes meiosis and pronuclei formation [15, 40–44]. In the present investigation, the abundance of *PLCZ1* was higher in the frozen-thawed semen of GF stallions, corroborating previous literature that reported higher mRNA transcript content in alive sperm together with a negative correlation with DNA fragmentation [40]. A low concentration of *PLCZ1* in subfertile stallions was previously reported [34,45] and is a feasible cause for low pregnancy rates after insemination with frozen-thawed semen from stallions in the PF group. In cattle, horse and men, higher abundance of *PLCZ1* in sperm was also associated with better ICSI success rates [40,46,47].

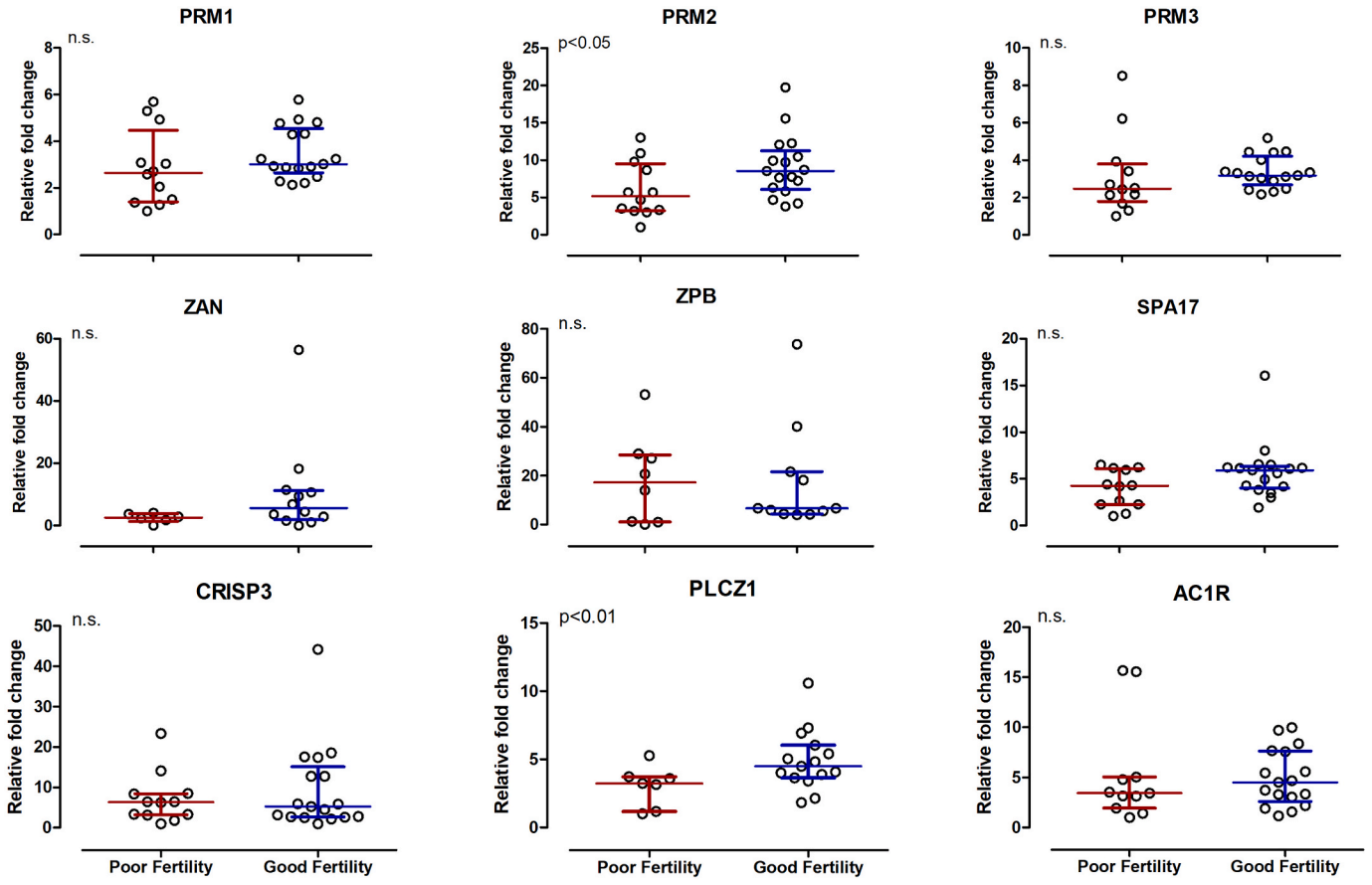

Fig. 1. Relative expression changes (calculated with the $2^{-\Delta\Delta CT}$ method; individual values plus interquartile range) in frozen-thawed semen from good fertility (blue) and poor fertility (red) stallions. Information for statistical differences between groups is given in the respective figure. Note different scales on y-axis. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Moreover, in the group of PF stallions and in the total sample, a positive correlation of *PLCZ1* with *SPA17* (*sperm autoantigenic protein 17*) was identified and tended to be present in GF stallions. The gene *SPA17* encodes for a sperm surface zona pellucida binding protein that was previously associated to fertility traits in stallions [14]. It has a central role in cell migration, adhesion and fertilization [48]. Finally, the abundance of *PLCZ1* was slightly positively correlated with that of the sperm membrane protein zonadhesin (*ZAN*). This unique molecule is localized close to the outer acrosomal membrane that gets exposed to the cell surface after sperm capacitation and for which differences in his polypeptide were identified between fertile and subfertile stallions [9,49,50]. It is worth noting that all genes linked in their expression levels among each other in this study are localized in the acrosomal and post acrosomal region and are therefore somehow involved in the fertilization process.

Into the group of transcripts that might cause male factor infertility, we included genes from the protamine family previously shown to be associated with altered semen parameters and embryo development in men and other species [2,51–54]. These nuclear proteins replace DNA-binding histones during late spermatogenesis. Whereas *PRM1* is present in all species with high amino acid homology, two variants of protamine-2 (*PRM2* and *PRM3*) have been determined in the stallion and constitute approximately 15 % of the total protamine content [51]. A reduced *PRM2/PRM1* mRNA ratio was previously described in raw semen, especially in spermatozoa with head defects [55]. In the present study, however, no differences in the *PRM2/PRM1* mRNA ratio in frozen-thawed semen of stallions with good and poor fertility were detected, but the abundance of *PRM2* mRNA was greater in the semen from good fertility stallions. Changes in the abundance of protamine transcripts during cryopreservation have previously been detected in stallion semen [56]. Results of the present study thus further support the idea of a crucial role of protamines with regard to the susceptibility of sperm to the cryopreservation process.

In disagreement with previous studies [6], no differences were identified in the mRNA abundance of *CRISP3* in the sperm of stallions with good and poor fertility. The *CRISP3* protein is, however, mainly of importance in equine seminal plasma where it prevents the phagocytosis of sperm by polymorphonuclear neutrophils (PMNs) in the uterus [57]. This trait is probably less important with regard to insemination with frozen-thawed semen. A previous study showed that seminal plasma removal causes increased binding of spermatozoa to PMNs [58], suggesting that the different abundance of this gene is given right from seminal plasma.

5. Conclusion

In conclusion, frozen-thawed sperm from stallions with higher *PRM2* and *PLCZ1* mRNA abundance are more likely to lead to successful fertilization. The results of the present study highlight the importance of further research into the significance of gene transcripts for male sub- and infertility in stallions and other species. Once techniques are improved with further study to detect all mRNAs of interest, it will be important to work towards developing a system with more robust fertility information to assess the utility of various mRNAs as biomarkers for fertility.

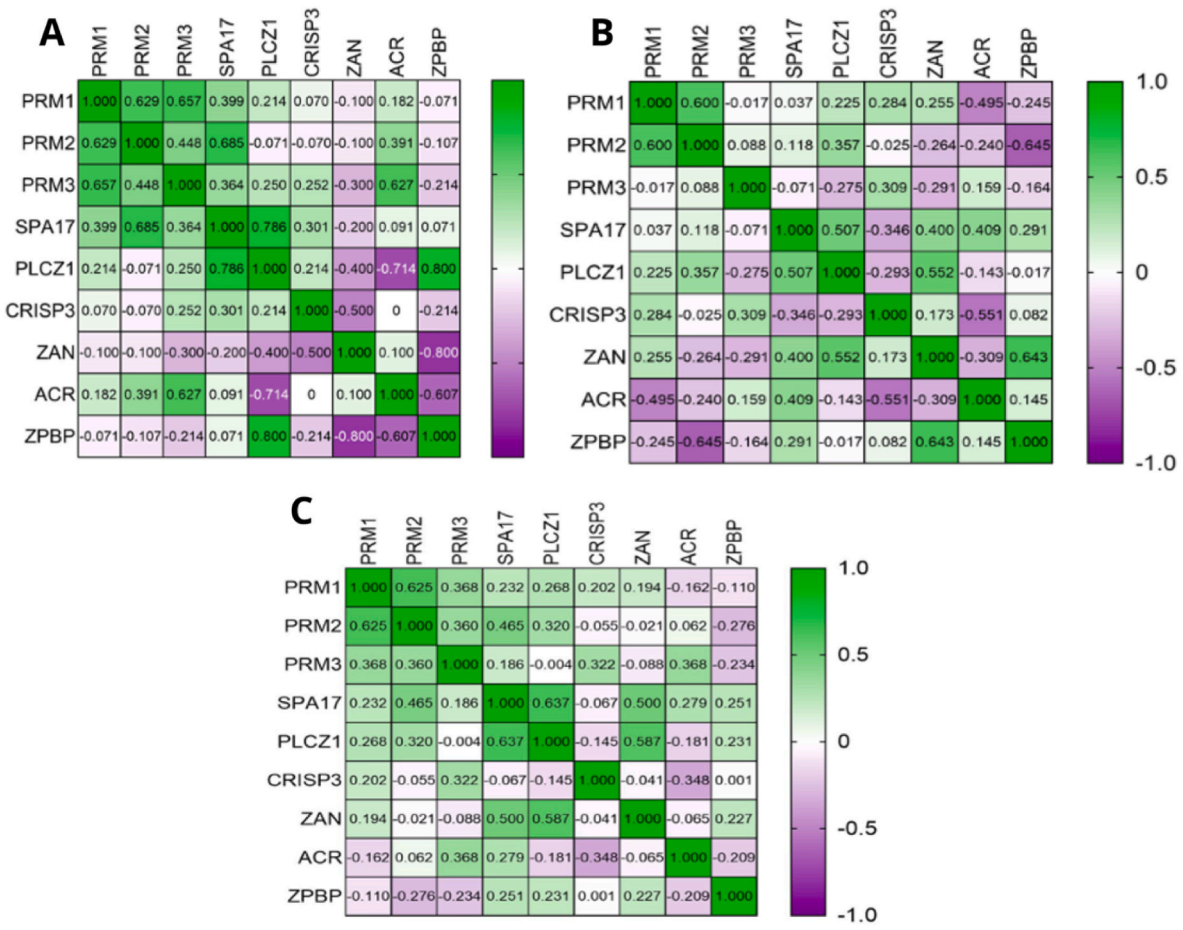

Fig. 2. Spearman correlations for gene expression. A: Spearman correlation coefficients for PF group; B: Spearman correlation coefficients for GF group; C: Spearman correlation coefficients for total sample (GF + PF). PRM1: protamine 1; PRM2: protamine 2; PRM3: protamine 3; SPA17: sperm autoantigenic protein 17; PLCZ1: phospholipase C zeta 1; CRISP3: cysteine-rich secretory protein 3; ZAN: zonadhesin; ACR: acrosine; ZPBP: zona pellucida binding protein.

Author declaration of interests

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Authorship

Christine Aurich, Maria Elena Falomo and Veronica Vigolo designed the study and performed the statistical analysis of data. Veronica Vigolo, Camille Gautier, and Reinhard Ertl executed the study and prepared the manuscript. All authors read, edited, and approved the final manuscript.

Ethical issues

According to the Austrian law for the protection of experimental animals (Law Decree n. 26 issued on 4 March 2014, art. 2), the approval by an ethical committee is not required under the circumstances that this trial was carried out. The owner's informed consent has been correctly collected.

CRediT authorship contribution statement

Veronica Vigolo: Conceptualization, Data curation, Formal analysis, Methodology, Validation, Writing – original draft, Writing – review & editing. **Camille Gautier:** Methodology, Validation. **Reinhard Ertl:** Methodology, Validation. **Christine Aurich:** Conceptualization, Formal analysis, Funding acquisition, Supervision, Visualization, Writing – review & editing. **Maria Elena Falomo:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – original draft.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2023.12.012.

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