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The structure of subpopulations of stallion spermatozoa after thawing differs between good and poor freezers

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Abstract: The aim of this study was to evaluate differences in the presence of sperm subpopulations in frozen-thawed semen in stallions with different freezability. The motility of individual spermatozoa of 24 stallions from 15 breeds was evaluated using computer-assisted sperm analysis (CASA) immediately after thawing (T0) and after 30 min of incubation (T30). In accordance with our previous studies, samples were initially divided based on their total motility into categories of good (GF) and poor (PF) freezers. *K*-means cluster analysis of kinematic parameters of spermatozoa was used to divide motile sperm (n = 57 630) into three subpopulations. Analysis of variance was used to evaluate differences in the subpopulations between GF and PF stallions at the times of incubation T0 and T30. Statistically significant differences were found in most kinematic parameters between PF and GF stallions as well as between the times of incubation T0 and T30 ($P < 0.05$). Spermatozoa of good freezers are represented more frequently in the fast and medium fast subpopulations and are faster and more linear than those of poor freezers ($P < 0.05$). Sperm from PF stallions were more strongly affected by longer incubation. The percentage of sperm in the fast and medium fast subpopulations was lower in samples from PF stallions, but assessment of the motility parameters in particular sperm subpopulations revealed that these sperm had good velocity. Poor freezer samples had lower sperm quality due to a reduced total proportion of motile sperm, and these samples were more sensitive to prolonged time after thawing. Thus, an efficient sperm selection method or a special insemination technique should be used for obtaining doses from stallions with poor freezability. Our study showed that the CASA system and cluster analysis are promising tools for better understanding the significant differences in the individual stallion freezability, and further research should be focused on their application in the field.

Keywords: reproduction; horse; cryopreservation; cluster analysis; CASA; fertility

It has been revealed in many species of mammals that the semen is not a homogeneous mixture of sperm but is composed of several populations with different kinematic characteristics (Martinez et al. 2006). The ratio and type of sperm subpopulations are not consistent and are influenced by physiological factors and processing during reproductive biotechnologies (Martinez-Pastor et al. 2011). The percentage of sperm subpopulations in the semen differs in individual stallions and between fresh and thawed sperm (Ortega-Ferrusola et al. 2009). The knowledge of the type and struc-

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ture of individual sperm subpopulations in semen is a possible tool that can help predict the fertilizing potential and freezability of a particular sample (Ortega-Ferrusola et al. 2009; Ferraz et al. 2014).

Determination of sperm motility is one of the most commonly used tests to evaluate the potential fertilizing capacity of semen (Graham 1996; Katila 2001; Kuisma et al. 2006). Nevertheless, as reported by Kuisma et al. (2006), no single standard test of stallion semen quality was consistently reliable for predicting the fertilizing capacity of the semen. The study shows the difficulty of frozen semen quality control in commercially produced stallion semen, and on the other hand, the difficulty of conducting fertility trials in horses. When computer-assisted sperm analysis (CASA) is available, the system is able to detect changes in the sperm motility more objectively (Amann and Waberski 2014), and particular kinematic parameters can describe the sperm motility in great detail (Simonik et al. 2015). However, determination of only total and progressive motility and even mean values of kinematic parameters may provide misleading results. Due to the heterogeneity of semen, it is therefore appropriate to focus on the evaluation of the percentage of different sperm subpopulations. Although it is known that the semen should be evaluated in relation to the represented sperm subpopulations, neither rules for the distribution of the sperm into subpopulations nor the number of subpopulations for individual animal species have been laid down (Simonik et al. 2015). To divide the sperm into subpopulations, appropriate statistical methods should be used, such as cluster analysis (Martinez-Pastor et al. 2011).

Applying the cluster analysis, the individual motile sperm are distributed into so called clusters characterised by their kinematic parameters. Based on them, individual subpopulations can be defined. In the semen of stallions, three and/or four sperm subpopulations have mostly been observed (Ortega-Ferrusola et al. 2009; Martinez-Pastor et al. 2011), defined as fast, slow, linear, and non-linear (Quintero-Moreno et al. 2003; Ortega-Ferrusola et al. 2009; Martinez-Pastor et al. 2011). Interestingly, differing fertilizing capacity was proved in males with similar average total motility but differing ratio of sperm subpopulations (Holt and Van Look 2004). Some types of sperm subpopulations are associated with the semen quality in stallions (Quintero-Moreno et al. 2003; Ortega-Ferrusola et al. 2009) and sperm fertilizing potential after thawing in bulls (Ferraz et al. 2014).

Cryopreservation in stallions, and the quality of insemination doses after thawing, is challenging because unlike in bulls, no selection for semen quality is performed, and there is a high variability between stallions in maintaining the fertilizing capacity of frozen-thawed semen (Ortega-Ferrusola et al. 2009; Sichtar et al. 2017) as well as between ejaculates of the same stallion (Janett et al. 2003). Based on the total or progressive sperm motility after thawing, stallions are divided into good and poor sperm freezers, hence their post-thaw fertilizing potential differs. In general, it is stated that 20–50% of stallions produce semen that cannot be frozen in a satisfactory way (Loomis and Graham 2008). However, so far it has not been elucidated whether the distribution of subpopulations differs between poor and good freezers. This knowledge of the semen heterogeneity may contribute to better prediction of male fertility, selection of a suitable assisted-reproduction method, and consequently lead to higher fertilizing capacity, with impact on the economic component of horse industry (Quintero-Moreno et al. 2003).

The aim of this study was to evaluate the presence of sperm subpopulations in the frozen-thawed semen from stallions with good or poor freezability and to verify the hypothesis that these subpopulations are differently represented in good and poor freezers.

MATERIAL AND METHODS

Semen collection and processing

In this study, ejaculates of 24 stallions were used from 15 different breeds. The collection of semen was performed in a certified equine reproduction centre (CZ 53790026, Equine Reproduction Centre Ltd., Pardubice-Mnětice, Czech Republic). Fractional collection of stallion ejaculates was performed using an open-ended type of artificial vagina with filter on the collecting vessel, to remove the gel part of the ejaculate and impurities. The collected sperm-rich fraction was immediately pre-diluted 1 : 1 with skim milk-based extender and centrifuged at 650 *g* for 15 minutes. After that, the supernatant was removed and sperm pellets were extended with Gent freezing extender, containing egg yolk and glycerol (Minitube, Tiefenbach, Germany). The final concentration of progressive spermatozoa was 350×10^6/ml. Extended sperm was packed

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into 0.5 ml straws and after 2 h of equilibration at 5 °C, horizontally frozen in a styrofoam box, 4 cm above the liquid nitrogen level for 15 min (Animal Reproduction Systems, Chino, CA, USA) and then stored in liquid nitrogen. Samples were thawed at 37 °C for 30 seconds. After thawing, the samples were diluted for the correct concentration for measurement in the CASA system with sp-Talp (114 mM NaCl; 3.2 mM KCl; 25 mM $NaHCO_3$; 0.34 mM $NaH_2PO_4 \cdot H_2O$; 10 mM sodium lactate; 2.0 mM $CaCl_2 \cdot 2H_2O$; 0.5 mM $MgCl_2 \cdot 6\ H_2O$; 10 mM HEPES, redistilled Milli-Q water).

Evaluation of sperm motility parameters

After thawing, motility parameters of frozen-thawed sperm samples were evaluated at T0 (after 5 min of pre-incubation at 37 °C in a water bath) and T30 (after 30 min of co-incubation at 37 °C in a water bath). Motility was evaluated using a computer assisted sperm analysis (CASA) system (Nis-Elements, v4.50; Laboratory Imaging, Prague, Czech Republic). A 4 µl drop of each semen sample was placed in a Makler chamber pre-warmed at 37 °C (Sefi-Medical Instrument, Haifa, Israel), and six fields per sample were evaluated at 100 × magnification using a phase-contrast microscope (Eclipse E600; Nikon, Tokyo, Japan) equipped with a heating plate pre-warmed at 37 °C. The evaluation was based on the analysis of 41 consecutive digitized images that were taken at a time lapse of 0.66 s with a camera at a frequency of 60 fps (DMK 23UM021; Imaging Source, Bremen, Germany). At least 200 trajectories were analysed per field. The following motility parameters were evaluated: total motility (TMOT, %), progressive motility (PMOT, %), amplitude of lateral head displacement (ALH, µm), beat-cross frequency (BCF, Hz), linearity (LIN, %), straightness coefficient (STR, %), average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s), and wobble (WOB, %). The spermatozoa were considered motile when VAP > 15 µm/s. The threshold values of STR and VAP for progressive motility were 50% and 30 µm/s, respectively.

Statistical analysis

Four datasets were created from the CASA-acquired data based on the incubation time and freezability of stallion semen. Freezability was determined based on the total motility (TMOT). Good freezer (GF) stallions were those that had 30% and more of total motile sperm, poor freezer (PF) stallions were those that had less than 30% of total motile sperm out of all evaluated samples. Only motile sperm were used for further analysis. Table 1 shows the number of sperm in individual categories (PF T0, PF T30, GF T0, and GF T30). A total of 57 630 spermatozoa were evaluated. The kinematic parameters were evaluated in the STATISTICA v12 software (StatSoft CR s.r.o., Prague, Czech Republic) using the *K*-means cluster analysis to distribute sperm into subpopulations. Euclidean distances were computed from the values of ALH, BCF, LIN, VAP, VCL, and VSL with 20 iterations into three (slow, medium fast, fast) subpopulations. To determine the differences in the distribution of these subpopulations, the chi-square test was used. One-way analysis of variance was used to determine differences within individual subpopulations between samples from stallions with poor and good freezability at the chosen incubation time after thawing. Significance was considered at $P < 0.05$ and results of individual kinematic parameters are presented as least-squares means ± standard error (LSM ± SEM) unless otherwise indicated below.

RESULTS

There was a significant difference between sperm samples from stallions with different freezability (good vs poor) and incubation time (T0 vs T30) in the distribution of sperm subpopulations and in the values of kinematic parameters after thawing ($P < 0.05$) (Figure 1 and Table 2).

Sperm percentages in subpopulations

Sperm subpopulations were divided into fast, medium fast and slow according to the mean of kinematic parameter values resulting from processing of data by cluster analysis (Table 3). The sperm percentages in the particular subpopulations differed between stallions with poor and good freezability ($P < 0.05$) (Figure 1). Poor freezer stallions had a lower percentage of spermatozoa in the fast and

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Table 1. Number of motile spermatozoa in individual categories

Group	Incubation time	Motile spermatozoa
PF	T0	5 706
	T30	11 309
GF	T0	25 461
	T30	15 154
Total count		57 630

GF = good freezers (n = 6); PF = poor freezers (n = 18); T0 = immediately after thawing; T30 = after 30 minutes of incubation

medium subpopulations and a higher percentage in the slow subpopulation than GF stallions (P < 0.05) at incubation times T0 and T30 (Figure 1).

Effect of stallion semen freezability on the occurrence of sperm subpopulations in the thawed semen

Table 2 shows resultant values of kinematic parameters of sperm motility in the tested groups within sperm subpopulations.

Incubation time T0

The fast sperm subpopulation of PF and GF stallions at incubation time T0 showed significant differences in all means of kinematic parameters (P < 0.05) except VSL. The values of ALH and LIN parameters in the fast subpopulation illustrated lower progressivity of the sperm of PF stallions than in GF stallions, but they were faster in all velocity (VCL, VSL, VAP) parameters.

The medium fast sperm population was characterised by high linearity (LIN) and by progressive sperm in both stallion categories. The sperm of GF stallions showed better linear motility (LIN) with higher oscillation (BCF) than in PF stallions (P < 0.05).

The slow sperm of both categories of stallion freezability had low linearity (LIN), were not progressive and had the lowest BCF of all subpopulations. All differences in the parameters of the slow subpopulation between PF and GF stallions were statistically significant (P < 0.05).

Incubation time T30

In the fast sperm subpopulation at time T30, all differences between the kinematic parameters for both categories of freezability were statistically significant (P < 0.05) except ALH. The values of VAP and VCL were lower in PF stallions, and in contrast, the values of VSL, BCF and LIN were higher.

The medium fast sperm of PF stallions were nonlinear, with the medium value of ALH and low BCF, while the medium fast sperm of GF stallions had a straight-line trajectory (LIN), with a low head amplitude (ALH) and high BCF (P < 0.05). Among the velocity parameters, VAP and VCL were higher in PF stallions and VSL was higher in GF stallions (P < 0.05).

The slow sperm subpopulation did not differ in ALH between PF and GF stallions; the other parameters showed significant differences (P < 0.05). The sperm of PF stallions were slower in all kinematic parameters compared to GF stallions.

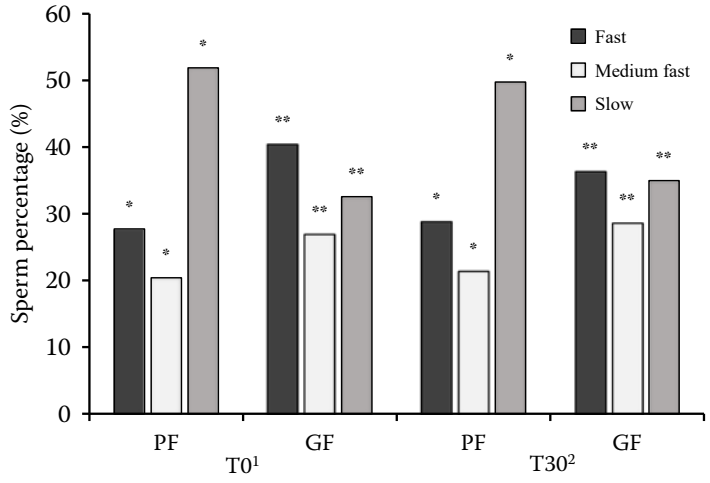

Figure 1. Sperm percentages in fast, medium fast and slow subpopulations of good and poor freezer stallions at incubation times T0 and T30
GF = good freezers; PF = poor freezers
[1,2]Different indices indicate significant differences between incubation times (T0, T30) in the particular groups of stallions (PF, GF) within the particular subpopulations (P < 0.05); *,**significant differences in individual subpopulations between poor freezers and good freezers at the same incubation time
The total number of motile spermatozoa analysed was 57 630. Motile spermatozoa in the particular datasets: PF T0 (n = 5 706), GF T0 (n = 25 461), PF T30 (n = 11 309), GF T30 (n = 15 154)

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Table 2. Kinematic parameters of sperm subpopulations defined by cluster analysis for distribution into three subpopulations (fast, medium fast, slow)

Subpopulation	Incubation time – freezability		ALH (μm)	BCF (Hz)	LIN (%)	VAP (μm/s)	VCL (μm/s)	VSL (μm/s)
Fast	T0	PF*	7.0 ± 0.0[1]	13.3 ± 0.1[1]	47.5 ± 0.3[1]	109.7 ± 0.4[1]	210.4 ± 0.9[1]	95.0 ± 0.4
		GF	6.3 ± 0.0[2*]	14.4 ± 0.1[2]	50.8 ± 0.1[2]	104.3 ± 0.2[2*]	189.2 ± 0.4[2*]	94.0 ± 0.2*
	T30	PF**	5.8 ± 0.0	17.5 ± 0.2[1]	61.6 ± 0.4[1]	95.9 ± 0.4[1]	154.1 ± 0.9[1]	90.3 ± 0.5[1]
		GF	5.9 ± 0.0**	14.7 ± 0.1[2]	50.9 ± 0.2[2]	98.5 ± 0.2[2**]	178.1 ± 0.4[2**]	88.1 ± 0.2[2**]
Medium fast	T0*	PF	3.7 ± 0.0	17.9 ± 0.2[1]	56.3 ± 0.4[1]	58.5 ± 0.5[1]	99.7 ± 1.0	54.2 ± 0.5[1]
		GF	3.7 ± 0.0	21.5 ± 0.1[2]	67.4 ± 0.2[2]	67.1 ± 0.2[2]	98.6 ± 0.5	64.4 ± 0.2[2]
	T30**	PF	5.6 ± 0.1[1]	9.6 ± 0.2[1]	30.6 ± 0.4[1]	68.8 ± 0.5[1]	169.8 ± 1.1[1]	49.5 ± 0.5[1]
		GF	3.6 ± 0.0[2]	20.3 ± 0.1[2]	65.9 ± 0.2[2]	63.8 ± 0.2[2]	95.9 ± 0.4[2]	61.2 ± 0.2[2]
Slow	T0*	PF	3.9 ± 0.0[1]	8.3 ± 0.1[1]	25.5 ± 0.2[1]	37.5 ± 0.3[1]	104.6 ± 0.7[1]	23.3 ± 0.3[1]
		GF	3.2 ± 0.0[2]	9.8 ± 0.1[2]	35.0 ± 0.1[2]	35.2 ± 0.2[2]	83.4 ± 0.4[2]	27.3 ± 0.2[2]
	T30**	PF	3.0 ± 0.0	9.3 ± 0.1[1]	32.9 ± 0.3[1]	28.2 ± 0.3[1]	72.2 ± 0.7[1]	22.1 ± 0.4[1]
		GF	3.0 ± 0.0	10.1 ± 0.1[2]	36.4 ± 0.2[2]	32.1 ± 0.2[2]	75.3 ± 0.4[2]	25.9 ± 0.2[2]

ALH = amplitude of lateral sperm head motion; BCF = beat cross frequency; GF = good freezers; LIN = linearity; PF = poor freezers; VAP = average path velocity; VCL = curvilinear velocity; VSL = linear velocity

[1,2]Different indices indicate a statistically significant difference between good and poor freezer stallions at the incubation times in the particular subpopulations (T0; T30) ($P < 0.05$); [*,**]different indices indicate significant differences between incubation times (T0, T30) in the particular groups of stallions (PF, GF) within the particular subpopulations ($P < 0.05$)

The total number of motile spermatozoa analysed was 57 630. Motile spermatozoa in the particular dataset: PF T0 (n = 5 706), GF T0 (n = 25 461), PF T30 (n = 11 309), GF T30 (n = 15 154)

Table 3. Values of kinematic parameters defined for particular sperm subpopulations (fast, medium fast and slow) in frozen-thawed stallion semen

Subpopulation	ALH (μm)	BCF (Hz)	LIN (%)	VAP (μm/s)	VCL (μm/s)	VSL (μm/s)
Fast	6.3 ± 0.3[a]	15.0 ± 1.6	52.7 ± 5.4	102.1 ± 2.5[a]	183.0 ± 13.1[a]	91.9 ± 2.3[a]
Medium fast	4.2 ± 0.3[b]	17.3 ± 1.6[a]	55.1 ± 5.4[a]	64.6 ± 2.5[b]	116.0 ± 13.1[b]	57.3 ± 2.3[b]
Slow	3.3 ± 0.3[b]	9.4 ± 1.6[b]	32.5 ± 5.4[b]	33.3 ± 2.5[c]	83.9 ± 13.1[b]	24.7 ± 2.3[c]

ALH = amplitude of lateral sperm head motion; BCF = beat cross frequency; LIN = linearity; VAP = average path velocity; VCL = curvilinear velocity; VSL = linear velocity

[a–c]Significant differences ($P < 0.05$) in the particular kinematic parameters between subpopulation types

The total number of motile spermatozoa analysed was 57 630

DISCUSSION

In this study, the effect of different freezability of stallion sperm on the distribution of motile sperm into subpopulations was investigated immediately after semen thawing and after 30-minute incubation. Samples were analysed by cluster analysis as a valuable statistical tool providing more detailed and relevant information about the sample heterogeneity (Ortega-Ferrusola et al. 2009). As confirmed by Nunez-Martinez et al. (2006) in their study on dogs, the use of the mean values hides actual differences in the sperm motility between individual males and also distorts the effect of biotechnological methods on the sperm, such as cryopreservation, and therefore the identification of subpopulations is a more adequate approach. Use of the cluster analysis either in the scientific community or for potential practical implications could lead to a great improvement in the prediction of sperm fertilizing capacity (Ferraz et al. 2014).

The present study also investigated the quality of frozen-thawed sperm samples of stallions, and the percentages of particular subpopulations were

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found to be significantly different between samples with poor and good freezability immediately after thawing and after 30-minute incubation. At both incubation times, the PF stallions had a lower percentage of the fast and medium fast subpopulations, while the percentage of the slow sperm was higher than in GF animals. In their study Ortega-Ferrusola et al. (2009) revealed that the cluster analysis of motility parameters obtained from CASA is able to find differences between stallions and ejaculates that remain hidden when traditional statistics of the same data have been used. Currently, in practice motility is predominantly evaluated subjectively, which means very limited information about the sperm ability to withstand cryopreservation.

It has been confirmed that the definition of sperm subpopulations in the semen substantially improves prediction of the semen freezability in stallions (Ortega-Ferrusola et al. 2009) as well as in dogs (Nunez-Martinez et al. 2006). Similarly to this study, Sichtar et al. (2017) showed that statistical evaluation and distribution of frozen-thawed sperm samples into subpopulations in the particular stallions with poor freezing capability enabled more precise detection of the percentage of sperm with higher values and hence with good-quality kinematic parameters (Sichtar et al. 2017). The limit for poor freezer stallions is that the percentage of these "good" spermatozoa in the semen is much lower than in good-freezer stallions whose TMOT is above 30% post-thaw.

In our study, we confirmed significant differences in the distribution of sperm to subpopulation between stallions with different freezability. Our results are consistent with studies of red deer (Ramon et al. 2012) and boars (Flores et al. 2009), in which the males with poor freezability were characterised by a high percentage of the sperm subpopulation with low kinematic parameters. Cryopreservation in boars is a long-term challenge (Jovicic et al. 2020), similarly like in stallions, because the sperm are highly sensitive to cold shock and both species have a high proportion of seminal plasma in the semen; studies conducted on boar sperm may therefore provide a comparison for our results in stallions. The mitochondrial activity was also lowest in poor freezer boars, and in addition, Hernandez et al. (2006) identified less homogeneous chromatin in PF boars.

In the present study, the 30-minute incubation both in GF and PF stallions had a statistically significant ($P < 0.05$) effect on the percentage of particular populations. In the study of Simonik and Sichtar (2018) on stallion frozen-thawed semen, the percentage of the fast sperm subpopulation was found to decrease after 30-minute incubation post-thaw, while the percentage of the slow subpopulation was found to increase. This finding agreed with the effect of incubation observed in our study on GF stallion sperm but not on PF stallion sperm. In the present study, the percentage of the fast subpopulation increased during the incubation in PF stallions and decreased in GF stallions.

The interpretation of the results of particular kinematic parameters indicates differences in the motility characteristics of sperm from stallions with different freezability in individual populations ($P < 0.05$). The sperm motility in GF stallions was more linear in most subpopulations than in PF stallions. Higher semen quality is associated with sperm displaying fast and linear motility and, on the other hand, slow non-linear sperm are associated with low semen quality (Quintero-Moreno et al. 2003; Ortega-Ferrusola et al. 2009; Martinez-Pastor et al. 2011; Ferraz et al. 2014).

Studies on mice have demonstrated that high VCL is essential for the passage through the uterotubal junction, and so for the sperm reservoir formation and penetration through the *zona pellucida* (Olds-Clarke 1996). This was also confirmed by the results of studies performed in men where fertile spermatozoa were obviously proved to reach higher VCL than the infertile ones (De Geyter et al. 1998). Ferraz et al. (2014), who used a bovine model, showed a significant and positive correlation of the kinematic parameter VCL with the number of sperm bound to *zona pellucida* and also found a relationship between the subpopulation with the fastest and progressively motile sperm of bulls and higher sperm quality and fertilizing capacity. In donkeys, Taberner et al. (2010) found a significant positive correlation between CASA parameters – VAP, VCL and ALH – and the *in vitro* conception rate. Farrell et al. (1998) also reported a strong correlation between several motility characteristics (BCF, LIN, VAP, STR and VCL) and *in vivo* fertility in cattle.

In the present study, sperm from GF stallions were expected to have higher mean values of velocity parameters, but in the fast subpopulation, the sperm of PF stallions had higher values of the kinematic parameters (VCL, VAP, ALH) than those of GF. Our results suggest that the sperm of PF stal-

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lions that have survived the cryopreservation process show high motility, but they are more strongly affected by the incubation time than the sperm of GF stallions. From this aspect, the correct timing of insemination with frozen sperm acquired from PF stallions seems important. The relationship between particular sperm subpopulations and *in vivo* fertility in horses is not clear; nevertheless, Gibb et al. (2014) accentuated a positive relationship between the sperm with fast motility and conception rate in mares. Hence, a positive relationship between the percentage of subpopulations with the highest values of velocity and fertilizing capacity in horses is assumed (Quintero-Moreno et al. 2003). As for cryopreservation, a higher percentage of the subpopulation with fast sperm in stallion semen is positively associated with higher freezability (Ortega-Ferrusola et al. 2009).

The number of motile sperm is lower in the insemination doses of PF stallions, but these sperm are relatively fast. The problem of the sperm quality of PF stallions does not therefore lie in the velocity of motile sperm, but in their total percentage in frozen-thawed semen and their sensitivity to the prolonged time of incubation after thawing. It is probable that besides motility, the sperm of PF stallions are also limited by their decreased ability to respond to changes in osmotic pressure and their plasma membrane integrity (Pukazhenthi et al. 2014). According to Hoffmann et al. (2011), the sperm of PF stallions show a lower tolerance to osmotic changes during cryopreservation in comparison with the sperm of GF stallions. The study of the motile sperm distribution into subpopulations may be a suitable tool to improve the current analyses of stallion semen quality providing a novel insight into its quality (Quintero-Moreno et al. 2003).

CONCLUSIONS

The sperm of GF stallions have a higher percentage of the fast subpopulation and the sperm in these subpopulations are mostly linear. The difference in the majority of motility parameters between GF stallions and PF stallions is significant. The sperm of PF stallions are more sensitive to a longer incubation time than the sperm of GF stallions.

To increase the quality of insemination doses and the success of subsequent insemination, a sperm selection method in PF stallions or a highly efficient method of insemination after thawing (e.g., the deep horn insemination technique) should be used. Further research in the field of CASA software modification is warranted, for example, programming of cluster analysis to proceed automatically after recording or implementation of artificial intelligence and machine learning to the CASA software, which can yield a more precise trajectory with possible better prediction values.

Conflict of interest

The authors declare no conflict of interest.

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