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Original article

Effect of the Freezing Month and Breed on Post-Thaw Equine Semen Quality

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Abstract

The quality of frozen-thawed semen in stallions is an important component of equine reproduction and can be influenced by various intrinsic and environmental factors. This study investigated the effects of stallion breed and freezing month on post-thaw semen quality. A total of 96 ejaculates were collected from Arabian, Arab-Barb, and French Saddle stallions and frozen between November 2023 and March 2024 using a stantardized cryopreservation protocol. Post-thaw semen quality was evaluated based on sperm motility, viability, morphology (head, midpiece, tail defects and cytoplasmic droplets), membrane integrity, and sperm head area. The freezing month significantly affected sperm motility and membrane integrity ($P < 0.05$), with the highest values recorded in January and March. Midpiece and tail defects, as well as cytoplasmic droplets, varied significantly across months ($P < 0.05$), whereas sperm viability and head area were not affected ($P > 0.05$). Breed had a significant influence on semen quality. Arab-Barb stallions showed lower sperm motility and higher rates of head and midpiece defects compared to Arabian and French Saddle stallions ($P < 0.05$). However, sperm viability and membrane integrity were not affected by breed ($P > 0.05$). In conclusion, both breed and freezing month influenced post-thaw semen quality. January appeared to be a particularly favorable period for semen cryopreservation. These findings highlight the importance of considering breed- and season-related factors when optimizing semen cryopreservation protocols to improve reproductive efficiency in stallions.

Keywords: Stallion Reproduction, Semen Cryopreservation, Post-Thaw Semen Quality, Sperm Motility, Sperm Morphology, Stallions

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INTRODUCTION

Stallion reproductive performance is shaped by a complex interplay of factors, among which breed and the seasonal conditions play a key role in determining semen quality and fertility potential (Najjar et al., 2017). Although equine spermatogenesis occurs continuously throughout the year, seasonal fluctuations in semen quality have been associated with changes in photoperiod, endocrine activity, and ambient temperature (Suliman et al., 2020). Additionally, breed-related differences have been shown to influence semen characteristicss and sperm cryotolerance (Kuhl et al., 2016). Variations in sperm membrane lipid composition, seminal plasma proteins, and metabolic activity among breed, may explain resistance to cryopreservation (Aurich et al., 2020).

Previous studies have reported that the month or season of semen collection and freezing may significantly influence post-thaw sperm quality. For example, post-thaw sperm motility and membrane integrity are often higher when semen is frozen during late winter or spring compared with other periods of the year (Suliman et al., 2020; Al Kass and Morrell, 2024). A retrospective evaluation of Warmblood stallions indicated significantly improved sperm motility and membrane integrity in samples frozen during the late winter and spring months, whereas sperm viability was lowest during the summer months (Sieme et al., 2008). Moreover, the interaction between breed and season may influence sperm cryosurvival, suggesting that some breeds may be more sensitive to seasonal variations than others (Greiser et al., 2020). Large-scale studies have further confirmed that both breed and collection month significantly affect seminal parameters such as sperm concentration, motile sperm count, DNA fragmentation, and post-thaw viability in different stallion breeds, including Warmblood, Arabian, Thoroughbred, and Quarter Horse stallions (Greiser et al., 2020; Oddi et al., 2021). However, information regarding these effects in North African equine populations, particularly under Tunisian breeding conditions, remains limited. This lack of data is especially relevant for Arabian, Arab-Barb, and French Saddle stallions, which are widely used in breeding and sport horse production in Tunisia.

Therefore, the present study investigated the effects of stallion breed (Arabian, Arab-Barb, and French saddle horse) and freezing month (November to March) on the quality of frozen-thawed semen. Specifically, we assessed sperm motility, viability, morphology, membrane integrity, and sperm head area after thawing. Understanding how these factors influence semen cryotolerance may help optimize cryopreservation protocols and improve reproductive efficiency in stallions.

MATERIALS and METHODS

Study Location

This study was conducted in northern Tunisia, in the city of Sidi Thabet, at the National Center for Equine Semen Production, part of the National Foundation for the Improvement of the Breed Horse. This center is one of the main facilities responsible for stallion semen collection and cryopreservation

in Tunisia. The region where located the center is characterized by a Mediterranean climate with mild winters and hot summers.

Animas and Semen Collection

A total of 96 ejaculates were collected from four Arab (n = 48), two Arab-Barb (n = 24) and two French Saddle (n = 24) stallions, and frozen between November 2023 and March 2024. Semen collection was carried out using a Hannover-type artificial vagina and a dummy mount (Haras Nationaux, 2004). The stallions were maintained under routine management conditions at the center, including controlled feeding, regular veterinary supervision, and standard housing conditions. Prior to inclusion in the freezing protocol, each stallion underwent a preliminary fertility evaluation. Only stallions meeting the following criteria were selected: fresh sperm motility above 70%, sperm concentration exceeding 100 × 10^6/mL, and total sperm count greater than 5 billion (Haras Nationaux, 2004).

Semen Crypreservation Protocol

Stallion semen cryopreservation was performed according to the stantardized protocol described by Haras Nationaux (2004). Semen samples underwent a dilution with INRA 96 (IMV Technologies, France) and were gradually cooled from 37 °C to 22 °C. The samples were then centrifuged at 600 × g for 10 min in order to remove seminal plasma. After centrifugation, the resulting sperm pellet was resuspended in the INRA 96 Freeze extender (IMV Technologies, France). The diluted semen was subsequently cooled to + 4 °C for 1 h 20 min to allow progressive temperature equilibration. During the cooling phase, semen was packaged into 0.5 mL straws. Each straw was previously labeled with the stallion name, breed, identification code, date of collection, ejaculate number, and production center. Filling and sealing of the straws were performed using an automatic straw filling and sealing machine. The filled straws were then placed in liquid nitrogen vapor at approximately 1–4 cm above the liquid–gas interface in a Digitcool programmable freezer. Finally, frozen straws were stored in liquid nitrogen at −196 °C until further analysis.

Post-thaw Semen Evaluation

Thawed semen quality was assessed between May and July 2024. For each ejaculate, two straws were randomly selected and thawed in a water bath at 37 °C for 30 s following standard thawing procedures (Haras Nationaux, 2004). After thawing, straws were wiped dry, and their contents were diluted in 2 mL of INRA96 extender.

Sperm motility (%) was evaluated immediately after thawing by placing a drop of semen between a slide and coverslip and observing under a phase-contrast microscope at 10× magnification with a heated stage (Najjar et al., 2025).

Sperm viability (%) was assessed using the VITA-EOSIN 'RAL' kit (1 × 100 mL Eosin – 1 × 100 mL Nigrosin; Reference: 608314), as decribed by Najjar et al. (2025). A mixture of 0.5 µL semen and

0.5 µL eosin was prepared in an Eppendorf tube and gently agitated for 30 seconds. Two drops of nigrosin were then added, and the mixture was spread on a slide to prepare a smear. Viability was determined by randomly counting 200 spermatozoa under a microscope at 40× magnification using a heated stage; pink-stained cells were classified as dead, while unstained ones were considered viable.

Sperm morphology defeacts was assessed using the same smear prepared for viability evaluation. Abnormal spermatozoa were classified according to criteria described by Samper (2009), including head defects, midpiece defects, tail defects, and cytoplasmic droplets (Table 1). A total of 200 cells were analyzed per smear, and the percentage of total abnormal sperm was determined.

Table 1. Classification of sperm morphological abnormalities evaluated in the study (Samper, 2009)

Category	Type of abnormality
Head defects	Macrocephalic head, microcephalic head, pyriform head, detached head
Midpiece defects	Bent midpiece, irregular midpiece
Tail defects	Coiled tail, bent tail, double tail
Cytoplasmic droplets	Proximal droplet, distal droplet

Sperm membrane integrity was evaluated using the hypo-osmotic swelling test (HOS), following the method of Colenbrander *et al.* (2003). A mixture of 0.4 µL semen and 0.4 µL hypotonic solution (100 mOsm), prepared according to Nie and Wenzel (2001), was incubated at 37°C for 20 minutes in a water bath. Following incubation, two drops of the mixture were placed on a slide and examined under a light microscope at 40× magnification. Spermatozoa exhibiting a coiled tail response, indicative of intact plasma membrane, were classified as HOS+ (%). A total of 200 cells were counted per drop (n = 400 sperm cells per sample).

Sperm head area was assessed through an open-source image analysis software ImageJ (version 1.54j, National Institutes of Health, USA). Head area (µm²) was calculated based on the pixel-defined boundaries of stained spermatozoa according to the method of Schneider *et al.* (2012) (Figure 1).

Figure 1. Representative micrograph of sperm heads used for morphometric analysis

Statistical Analysis

Data on semen characteristics were analysed using SAS software (SAS Institute Inc., version 2005). Analysis of variance (ANOVA) using the General Linear Model (GLM) procedure was performed to assess the effects of freezing month and stallion breed on post-thaw semen quality parameters, according to the statistical procedure described by SAS Institute (2005). Means were compared using the Student–Newman–Keuls (SNK) test. Statistical significance was considered at $P < 0.05$.

RESULTS and DISCUSSION

The results of sperm motility, viability, membrane integrity and head area are presented in Table 2. Sperm motility (%) and the percentage of spermatozoa with intact plasma membrane (HOS+) varied significantly with the freezing month ($P < 0.05$). The highest values were observed in January (55.3% and 48.9%) and March (49.6% and 55.6%), whereas the lowest values were recorded in November (29.9% and 37.3%) and December (36.3% and 36.5%), while February showed intermediate levels (40.0%).

Sperm viability did not differ ($P > 0.05$) between months. However, higher values were observed in January (50.7%) and February (49.6%), while lower values were recorded in November (38.6%) and March (38.6%).

Similarly, analysis of sperm head area across freezing months showed no significant variation ($P > 0.05$). Mean value ranged from 11.3 µm² in November to 14.0 µm² in March.

Table 2. Variation of semen characteristics according to the freezing month (Means ± SD)

Characteristics	November 2023 (n = 28)	December 2023 (n = 18)	January 2024 (n = 20)	February 2024 (n = 16)	March 2024 (n = 15)	P-value
Sperm motility (%)	29.9 ± 3.6^{b}	36.3 ± 6.2^{b}	55.3 ± 4.5^{a}	40.0 ± 4.2^{ab}	49.6 ± 7.8^{a}	0.0001
Sperm viability (%)	38.6 ± 2.5	40.8 ± 3.6	50.7 ± 5.0	49.6 ± 6.9	38.6 ± 2.5	0.2465
Sperm with intact plasma membrane HOS+ (%)	37.3 ± 2.8^{b}	36.5 ± 2.2^{b}	48.9 ± 4.5^{a}	40.1 ± 4.4^{ab}	55.1 ± 8.0^{a}	0.0406
Sperm area (µm^2)	11.3 ± 1.5	13.8 ± 1.3	13.2 ±1.2	12.9 ± 1.1	14.0 ± 0.7	0.2277

^{a,b} Different superscript letters within a row indicate statistically significant differences between months ($P < 0.05$).

Sperm abnormalities are presented in Table 3. Statistical analysis showed that midpiece defects, tail defects, and cytoplasmic droplets varied with freezing months ($P < 0.01$), while head defects did not vary ($P > 0.05$). Midpiece defects increased significantly in March (6.7%). Tail defects were highest in February (15.0%) compared with January (8.5%). Cytoplasmic droplets decreased gradually from November (1.8%) to March (0.0%).

Table 3. Variation of sperm abnormalities according to the freezing month (Means ± SD)

Abnormalities	November 2023 (n = 28)	December 2023 (n = 18)	January 2024 (n = 20)	February 2024 (n = 16)	March 2024 (n = 15)	P-value
Head defects (%)	7.4 ± 0.9	7.7 ± 1.4	7.4 ± 2.9	6.0 ± 1.3	6.7 ± 0.9	0.6555
Midpiece defects (%)	6.0 ± 0.8[a]	4.7 ± 1.2[a]	5.1 ± 0.7[a]	2.0 ± 0.2[b]	6.7 ± 0.8[a]	0.0001
Tail defects (%)	14.5 ± 1.0[a]	11.5 ± 1.2[a]	8.5 ± 1.4[b]	15.0 ± 1.1[a]	10.7 ± 1.3[b]	0.0022
Cytoplasmic droplets (%)	1.8 ± 0.3[a]	1.0 ± 0.3[a]	0.6 ± 0.2[b]	0.2 ± 0.1[b]	0.0 ± 0.0[b]	0.0001
Total abnormal sperm (%)	29.5 ± 2.0[a]	24.8 ± 3.0[b]	21.6 ± 3.9[b]	23.1 ± 1.7[b]	24.0 ± 2.2[b]	0.0045

[a,b] Different superscript letters within a row indicate statistically significant differences between months (P < 0.05).

Regarding the breed effect, the results of sperm motility, viability, membrane integrity and head area are presented in Table 4. Statistical analysis showed that stallion breed had a significant effect on sperm motility ($p<0.05$), while no significant differences were observed among breeds for sperm viability, plasma membrane integrity (HOS+), or sperm head area ($P > 0.05$). Arab-Barb stallions displayed the lowest sperm motility (26.3 ± 6.4%), which was significantly lower than that of Arabian (39.6 ± 2.7%) and French Saddle stallions (34.0 ± 11.2%).

Table 4. Variation of semen characteristics according to the stallion's breed (Means ± SD)

Characteristics	Arab (n = 48)	Arab-Barb (n = 28)	French Saddle (n = 24)	P-value
Sperm motility (%)	39.6 ± 2.7a	26.3 ± 6.4b	34.0 ± 11.2a	0.0288
Sperm viability (%)	42.7 ± 2.0	38.7 ± 5.7	53.6 ± 9.7	0.9196
Sperm with intact plasma membrane HOS+ (%)	41.0 ± 1.9	34.3 ± 6.9	43.5 ± 11.9	0.8340
Sperm area (µm2)	13.0 ± 0.7	14.5 ± 0.5	14.0 ± 1.0	0.2134

[a,b] Different superscript letters within a row indicate statistically significant differences between breeds (P < 0.05). HOS+ (%) indicates the percentage of spermatozoa exhibiting a coiled tail response, indicative of intact plasma membrane.

Breed had a significant effect on sperm morphology (Table 5), especially regarding head defects, midpiece defects, and total abnormal spermatozoa (P < 0.05). Head defects were significantly (P < 0.01) more prevalent in Arab-Barbs (14.2%) than in Arabian (6.9%) or French Saddle stallions (2.0%). Similarly, midpiece defects were significantly (P < 0.05) more common in Arab-Barbs (10.0%) than in the other groups of breed.

Total sperm abnormalities followed significantly the same trend (P < 0.01): Arab-Barbs had the highest total abnormal sperm rate (39.2%), compared to Arabian (16.0%) and French saddle horses

(12.0%). In contrast, the rates of cytoplasmic droplets and tail defects did not differ significantly between breeds.

Table 5. Variation of sperm abnormalities according the stallion's breed (Means±SD)

Abnormalities	Arab (n = 48)	Arab-Barb (n = 28)	French Saddle (n = 24)	P-value
Head defects (%)	6.9 ± 0.8^{b}	14.2 ± 1.6^{a}	2.0 ± 0.8^{c}	0.0026
Midpiece defects (%)	4.8 ± 0.6^{b}	10.0 ± 1.9^{a}	4.6 ± 0.4^{b}	0.0133
Tail defects (%)	13.3 ± 0.7	13.0 ± 2.5	3.0 ± 0.7	0.1470
Cytoplasmic droplets (%)	1.1 ± 0.2	2.0 ± 0.3	2.2 ± 0.5	0.0866
Total abnormal sperm (%)	16.0 ± 1.5^{b}	39.2 ± 3.4^{a}	12.0 ± 1.5^{b}	0.0092

[a,b] Different superscript letters within a row indicate statistically significant differences between breeds ($P < 0.05$).

In the present study, seasonal variation significantly affected post-thaw sperm motility and plasma membrane integrity. The higher sperm motility and membrane integrity (HOS+) in January and March suggest that environmental and physiological conditions during this period may favor optimal sperm quality, likely reflecting the transition from mid winter toward the onset of the breeding season. Photoperiod, ambient temperature, and testicular endocrine activity are known to influence testicular function in stallions. Stallion spermatogenesis follows seasonal patterns; and semen collected during winter and early spring has frequently been reported to exhibit improved sperm motility and membrane integrity under stable management and environmental conditions (Aurich, 2016; Crespo et al., 2020; Dziekonska et al., 2025). Seasonal fluctuations in testosterone and other endocrine factors may influence both spermatogenesis and sperm membrane composition. During late winter and early spring, the progressive increase in photoperiod and moderate environmental temperatures may contribute to enhance sperm membrane stability and cellular metabolism, thereby improving semen quality (Schmidt et al., 2017). Conversely, the reduced values observed in November and December likely reflect a transitional phase toward the nonbreeding season, when testicular activity gradually declines. Sperm motility is often one of the first semen parameters affected by seasonal changes in spermatogenic activity, preceding alterations in sperm concentration or morphology (Sieme et al., 2008). In contrast, sperm viability did not vary significantly across freezing months, suggesting relative stability of this parameter during the study period. This stability may be attributed to consistent management, standardized collection procedures, and controlled cryopreservation protocols that reduce environmental variability (Sieme et al., 2008). Similar findings were reported by Aurich et al. (2020), who noted that sperm viability is often less sensitive to seasonal changes than sperm motility under controlled conditions. Nevertheless, the tendency toward higher sperm viability during midwinter months may still have biological relevance. Improved testicular function and favorable hormonal profiles during this

period may enhance membrane resilience and resistance to cryodamage (Ortega-Ferrusola et al., 2017). Sperm morphometric characteristics also appeared stable throughout the study period. The absence of significant variation in sperm head area accross freezing months suggests that sperm morphometry is largely unaffected by short-term environmental changes. This observation is consistent with previous studies indicating that sperm head dimensions are primarily determined during spermatogenesis and are strongly influenced by genetic and breed-related factors rather than seasonal conditions (Banaszewska et al., 2015). Similar results have been reported in stallions and other domestic species, where morphometric parameters exhibit greater inter-individual than seasonal variability (Ortega-Ferrusola et al., 2017).

Despite the stability of morphometric traits, some sperm morphological abnormalities varied with freezing month. Midpiece defects increased significantly from February to March, while tail defects were higher in February. These variations may reflect seasonal influences on spermiogenesis and mitochondrial development, which are critical for sperm motility (Suliman et al., 2020). Similar observations have been reported in stallions, where morphological defects vary with photoperiod and changes in testicular activity (Sieme et al., 2008).

A gradual decrease in cytoplasmic droplets from November to March was also observed, suggesting progressive improvement in sperm maturation as the breeding season approaches. Cytoplasmic droplets are typically associated with incomplete epididymal maturation; therefore, their reduction may reflect enhanced epididymal function during late winter and early spring (Wach-Gygax et al., 2017). In this regards, environmental conditions play an important role in stallion reproductive physiology. For instance, Kandiel and El Khawagah (2018) reported that high summer temperatures can negatively affect stallion fertility through alterations in hormonal secretion and semen characteristics. Given the strong relationship between morphologically normal sperm and stallion fertility (Whitesell et al., 2019; Orsolini et al., 2021), semen collected in late winter and early spring is generally associated with improved sperm morphology, motility, and cryosurvival, whereas samples obtained in autumn and early winter often exhibit increased morphological defects and reduced post-thaw survival (Sultan et al., 2023).

Breed also significantly influenced semen quality parmeters in the present study. Arab-Barb stallions exhibited lower sperm motility compared with Arabian and French Saddle stallions. This reduced sperm motility may be associated with the higher incidence of midpiece and tail abnormalities found in the Arab-Barb group, as structural defects in these spermatozoa parts can compromise flagellar function and mitochondrial activity (Morrell et al., 2008; Ortega-Ferrusola et al., 2017).

Breed differences were also evident in sperm morphology. Arab-Barb stallions showed significantly higher proportions of head defects, midpiece defects, and total abnormal spermatozoa compared with Arabian and French Saddle stallions. Similar breed-related differences in semen quality

have been reported previously in stallions (Greiser et al., 2020; Sudhakar et al., 2021). The higher prevalence of head defects in Arab-Barb stallions may be associated with disturbances during spermiogenesis or chromatin condensation, potentially related to genetic background (Gonçalves et al., 2023). Likewise, the increased occurrence of midpiece defects may negatively affect sperm motility due to impaired mitochondrial function and retuced ATP production.

In contrast, sperm viability, membrane integrity (HOS+), and sperm head area did not differ significantly between breeds. These results support previous observations suggesting that these characteristics are less influenced by breed than functional sperm parameters (Morrell et al., 2010).

Conclusion

This study showed that both freezing month and breed influence post-thaw semen quality. Seasonal variation mainly affected sperm motility and membrane integrity, whereas sperm morphometric traits remained relatively stable across freezing months. In addition, Arab-Barb stallions exhibited lower sperm motility and higher rates of morphological abnormalities compared with Arabian and Frensch Saddle stallions. These findings highlight the importance of considering both seasonal and genetic factors when optimizing semen collection and cryopreservation strategies in equine breeding programs. Further research is warranted to better understand the physiological mechanisms underlying these variations and their implications for stallion fertility.

Additional Declaration

Author Contributions

First author: Conceptualization, methodology, data curation and writing the original draft. Second author: Data curation and statistical analysis. Third author: Methodology. Fourth author: Conceptualization.

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Responsible Artificial Intelligence Statement

In this study, artificial intelligence tools were used in language editing. The artificial intelligence tool was used to correct language errors. We declare that we, as the authors, take full responsibility for the problems that may arise from the content produced by artificial intelligence.

Conflicts of Interest

The authors declare that there are no conflicts of interest related to the publication of this study.

Ethics Approval

In all processes of this study, the principles of Pen Academic Publishing Research Ethics Policy were followed.

Ethical approval was not required for this study because semen samples were collected as part of routine breeding management and did not involve any invasive procedures or experimental manipulation of the animals. All procedures complied with Tunisian legal requirements (Livestock Law No. 2005-95 of October 18, 2005).

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