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Evaluation of the variations in spermatological parameters of Arabian stallions according to different periods of breeding season

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ABSTRACT

In this study, determining of the variations on spermatological characteristics of Arabian stallion semen in the different periods of the breeding season is aimed. The periods have been described as the beginning of the season (BS: 15th February – 15th March), mid-season (MS: 15th April – 15th May), and end of the season (ES: 30th May – 30th June). A total of 7 Arabian stallions that were 5-10 years old, with body condition scores between 3-3.5 and without any problems in terms of general and reproductive health, were enrolled in the study. In total, three semen samples were collected from each stallion, once in each period. Then, these samples were evaluated in terms of volume, concentration, total motility (TMOT), progressive motility (PMOT), viability, high mitochondrial membrane potential (HMMP), acrosome integrity (AI), capacitation index (Ci), and lipid peroxidation (LPO) parameters, which were analyzed by computer assisted sperm analysis and flowcytometry devices. As a result, the differences in volume, concentration, TMOT, and PMOT between the BS, MS, and ES periods were statistically insignificant ($p>0.05$). The lowest viability values were determined in the BS period ($p<0.001$). The highest values of the HMMP were obtained in the MS ($p<0.05$). The AI results were higher in the ES period than the others ($p<0.05$). While the Ci ($p<0.01$) and LP ($p<0.001$) parameters had the highest values in the BS period, a gradual decrease has been observed until the ES period. In conclusion, it has been observed that the best semen quality can be obtained in the period of MS (15 April – 15 May) for young (5-10) aged Arabian stallions breeding in Türkiye.

Arap aygırlarında spermatolojik parametrelerin sezonun farklı dönemlerine göre değişimlerinin değerlendirilmesi

ÖZET

Bu çalışmada, Arap aygırlarında aşım sezonu içerisinde farklı dönemlerde spermatolojik parametrelerde gözlemlenen farklılıkların belirlenmesi amaçlanmıştır. Aşım sezonu içerisindeki farklı dönemler sezon başı (SB: 15 Şubat – 15 Mart), sezon ortası (SO: 15 Nisan – 15 Mayıs) ve sezon sonu (SS: 30 Mayıs – 30 Haziran) olarak belirlenmiştir. Yaşları 5-10 arasında değişen, vücut kondisyon skorları 3-3,5 arasında olan, genel ve reprodüktif sağlık durumları açısından herhangi bir problem olmayan toplam 7 Arap aygırı çalışmaya dahil edildi. Her bir aygırdan, her bir dönemde bir defa olmak üzere, toplam 3 sperma alma işlemi gerçekleştirildi. Alınan spermalar miktar, yoğunluk, toplam motilite (TMOT), progresif motilite (PMOT), canlılık, akrozom sağlamlığı (AI), yüksek mitokondriyal membran potansiyeli (HMMP), kapasitasyon indeksi (Ci) ve lipid peroksidasyon seviyesi (LPO) parametreleri açısından bilgisayar yardımlı sperma analizi ve akış sitometri yöntemleriyle analiz edildi. Analiz sonuçlarına göre miktar, yoğunluk TMOT ve PMOT parametrelerinde periyotlar arasındaki farklılıklar istatistiksel olarak benzer bulundu ($p>0,05$). En düşük canlılık değeri SB periyodunda elde edildi ($P<0,001$). En yüksek HMMP değeri SO periyodunda bulundu ($p<0,05$). Akrozom sağlamlığı değeri SS periyodunda diğer periyotlara göre daha yüksekti ($p<0,05$). Kapasitasyon indeksi ($p<0,01$) ve LPO ($p<0,001$) değerleri SB periyodunda en yüksek değerlere sahipken, SS periyoduna gidildikçe kademeli olarak düşüş gösterdi. Sonuç olarak, Türkiye'de yetiştiriciliği yapılan genç yaşlı (5-10) Arap aygırlarında en iyi sperma kalitesinin SO periyodunda (15 Nisan – 15 Mayıs) alınabileceği sonucuna ulaşılmıştır.

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1. Introduction

Horses are species that have the ability to restrict or increase their reproductive performance on particular days of the year in order to grow new generations in a healthy way (1). The cyclic patterns of reproductive activities in horses are mainly determined by day length (2). Seasonal dependence in horses occurs clearly in mares, while it is not certainly observed in stallions. On the other hand, decreases in testosterone concentration, gametogenesis, functions of reproductive organs, and semen quality are marked in the non-breeding season in stallions. Accordingly, there is a significant decrease in the fertility of stallions out of the breeding season (3, 4). However, some researchers have reported that stallions are fertile throughout the year, although there are small fluctuations in their sexual activities, especially in the region between 30^{o} and 40^{o} Northern latitudes, including Türkiye (1, 5, 6). There is still conflicting information on seasonal changes in the reproductive status of stallions (7).

It is reported that the reproductively active period of stallions is generally seen between January and October in the Northern Hemisphere (4, 8), and the period with the highest reproductive activity is indicated as the period between late spring and early summer (9). In light of this information, the period between February and June (15th February – 30th June) has been officially determined as the breeding season in horse breeding enterprises in the Northern Hemisphere. On the other hand, foals born in the same year must participate in the same age groups in the competitions. This situation forces breeders to ensure that their mares conceive as early as possible from the beginning of the season (1). However, it is obvious that different levels of reproductive activity would be encountered in stallions during the breeding season, which includes different seasons and day lengths. On the other hand, there is no study on the changes in spermatological parameters in Arabian stallions during different periods of the breeding season. In a study, it was concluded in the morphological and functional evaluations of semen belonging to different stallion breeds that the parameters measured in different periods during the breeding season had different values between January-March, March-May, and May-July, and the best values were obtained from the semen collected between March-May (1). In the case of Arabian horse stallions, such an evaluation has not yet been made. It is important to reveal the results of comprehensive spermatological examinations in different periods during the breeding season in Arabian horse stallions, which are the most bred breed in Türkiye.

In this study, it is aimed to reveal the variations in spermatological characteristics of Arabian stallion semen in the different periods of the breeding season, such as the beginning of the season (BS: 15th February – 15th March), mid-season (MS: 15th April – 15th May), and end of the season (ES: 30th May – 30th June). For this purpose, differences and similarities in semen volume, concentration, total motility (TMOT), progressive motility (PMOT), viability, high mitochondrial membrane potential (HMMP), acrosome integrity (AI), capacitation index (Ci), and lipid peroxidation (LP) parameters will be determined in Arabian stallions in the mentioned periods.

2. Material and Methods

In the study, a total of 7 Arabian stallions, between 5-10 years old, with body condition scores between 3-3.5, and without any problems in general and reproductive health conditions, were used. In total three semen samples were collected from each stallion, once at each period which were described as BS (15th February – 15th March), MS (15 April – 15 May), and ES (30 May – 30 June) via INRA model artificial vagina. Firstly, semen samples were evaluated in terms of volume (ml). Then, these samples were evaluated by a computer assisted semen analyzer (CASA, SCA, Microptic, Spain) in terms of concentration (x10^{6}/ml), TMOT (%) and PMOT (%). Viability (%), HMMP (%), AI (%), Ci (%), and LP (%) parameters were analyzed by flowcytometry (Beckmann Coulter, USA).

Semen collections from the stallions were made in the presence of a mare in estrus to induce sexual and mounting activity. After the stallions mounted on the mare, semen was collected using an INRA model artificial vagina with an in-line gel filter. Gel-free semen samples were transferred to pre-warmed graduated tubes, and their volumes were determined and recorded. Then, 100 µl of the fresh semen was diluted 1:10 (v:v) with the TRIS extender (2.44 g TRIS; 1.36 g Citric Acid; 0.82 g Glucose; 100 ml distilled water) before the CASA analysis to obtain a suitable

concentration for the analysis. After dilution, 5 µl of the sample was placed on a slide and covered with a cover slide. At least seven randomly chosen microscopic fields were analyzed in each semen sample on a phase contrast microscope with a heating plate (Nikon ECLIPSE 50i) connected to the system, accompanied by a camera (Basler) at 100x magnification. In the analysis, concentration (x10^6/ml), TMOT (%), and PMOT (%) parameters were evaluated.

For flowcytometric analysis, a 5 µl semen sample was diluted with Phosphate Buffer Solution (PBS) to have a concentration of 2.5 million spermatozoa/ml. An appropriate amount of sample was taken from the diluted semen for each analysis method, and the appropriate amount of fluorochrome dye was added according to the analysis procedure. Then, the prepared semen-dye mixtures were incubated at 38 ^oC for 30 minutes, and they were analyzed by a flowcytometry device (Beckman Coulter, Fullertone, CA, USA) via CytExpert 2.2 software (Beckman Coulter, Fullerton, CA, USA) connected to the system. At least 10,000 spermatozoa were counted, and a forward scatter area (FSC-A) versus side scatter area (SSC-A) density plot was used to exclude doublets (SPERM); doublet exclusion was further verified (TARGET) through a forward scatter area (FSC-A) versus forward scatter height (FSC-H) plot (Figure 1).

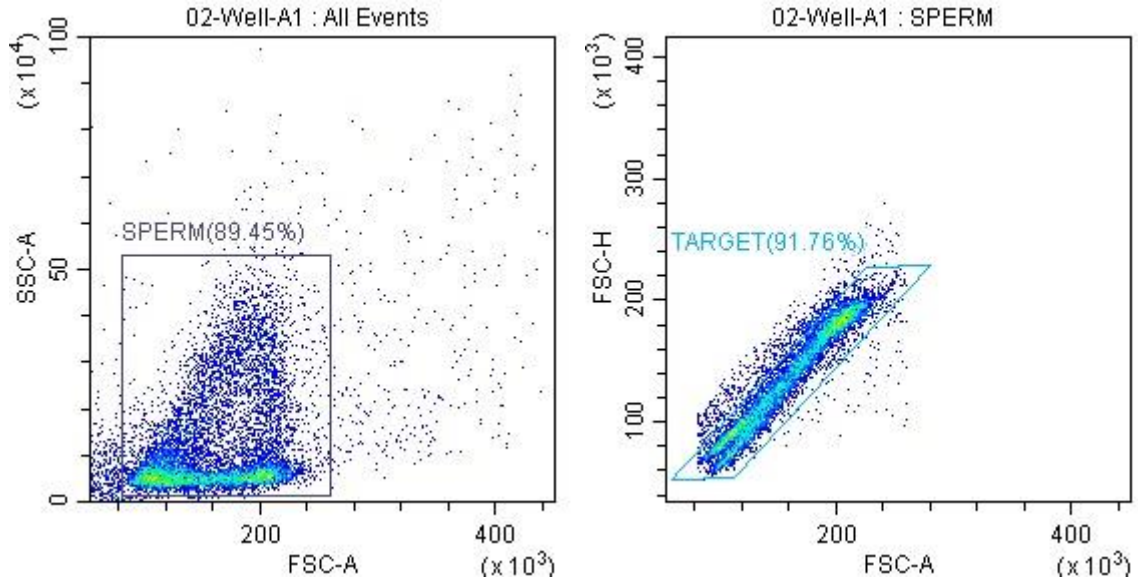

Figure 1: Exclusion of doublets by forward and side scatter areas of counted beams (left side, SPERM) and verification of exclusion of doblets from SPERM by area versus height of forward scattered beams (right side, TARGET)

Şekil 1: *Sayılan partiküllerde ileri ve yan yüzey alanlarına göre çift sayımların çıkartılması (sol taraf, SPERM) ve SPERM içerisinde ileri yüzeylerin alan ve yüksekliklerine göre çıkarma işlemlerinin doğrulaması (sağ taraf, TARGET)*

Viability was determined with the LIVE/DEAD Sperm Viability Kit (L7011, ThermoFisher). 246 µl of diluted semen was taken and transferred to 96 microplate wells, and 2.5 µl of SYBR-14 and 1.5 µl of PI dye were added. Viable (SYBR+/PI-), moribund (SYBR+/PI+), dead (SYBR-/PI+), and non-stained (SYBR-/PI-) spermatozoa populations were determined in the histogram formed after the analysis (Figure 2a). In statistical analysis, only the percentage of viable (SYBR+/PI-) spermatozoa population was evaluated (10).

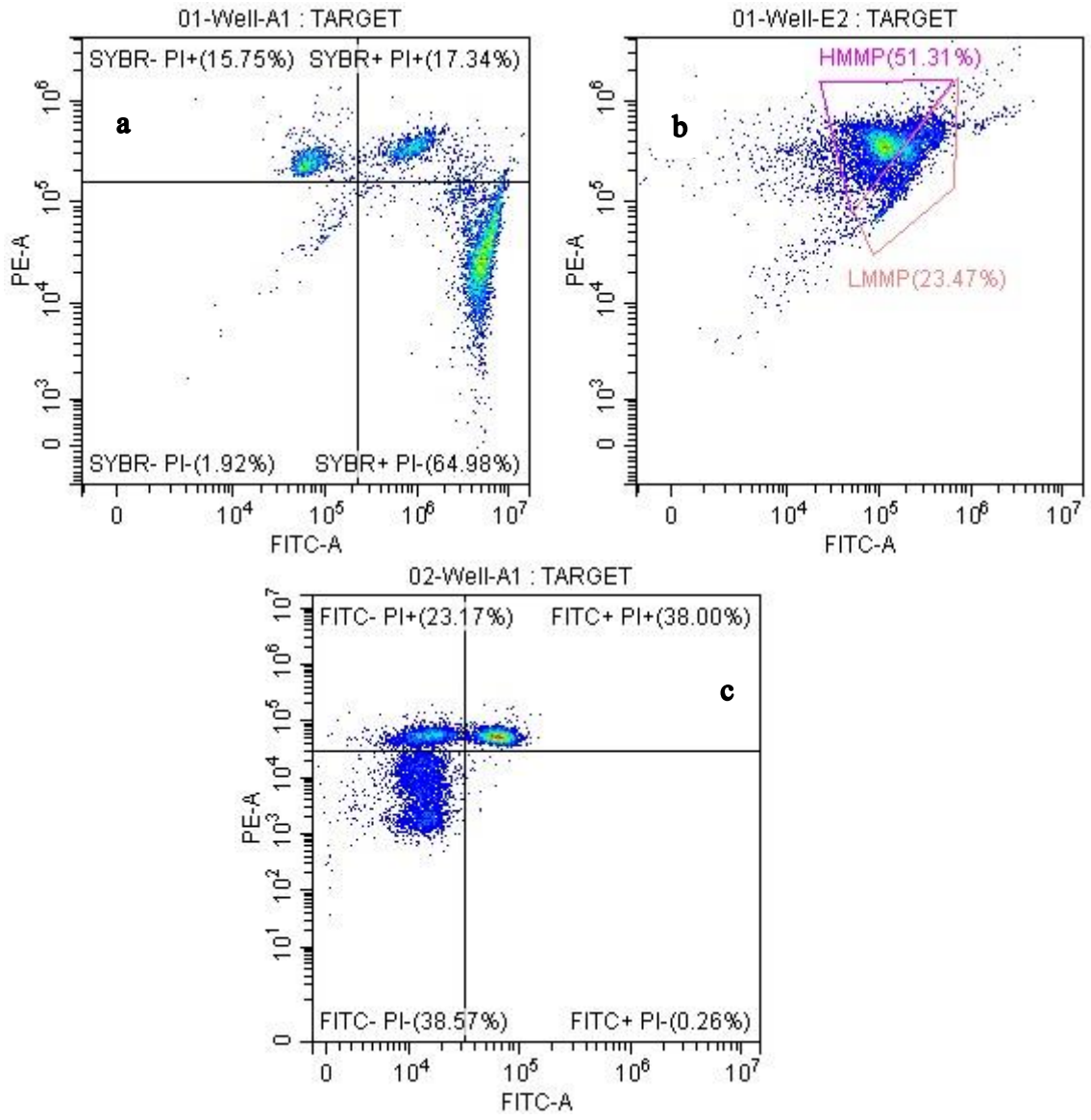

Figure 2: Determination of viability (a), HMMP (b) and AI (c) by gating on FITC vs. PE density plot chart.
Şekil 2: *FITC ve PE düzlemlerinin yoğunluk grafiğine göre Canlılık (a), HMMP (b) ve AI (c) analizlerinde kapılama işlemleri.*

Mitochondrial activity parameter was determined with the MitoProbe™ JC-1 Assay Kit (M34152, ThermoFisher). 247.5 µl of the diluted semen was transferred to the wells of 96 microplates, and 2.5 µl of JC-1 dye was added. In the histogram formed after the analysis, populations of spermatozoa with high mitochondrial activity (HMMP, orange fluoresce) and low mitochondrial activity (LMMP, green fluoresce) were determined (Figure 2b). The percentage of HMMP spermatozoa population was used in statistical analyses (7).

The AI parameter was determined by FITC-PNA/PI (V13242, ThermoFisher). 246 µl of diluted semen was transferred to the microplate wells, and 2.5 µl of FITC-PNA and 1.5 µl of PI dye were added. Viable and intact acrosome (FITC-/PI-), viable and acrosome damaged (FITC+/PI-), dead and acrosome intact (FITC-/PI+), and dead and acrosome-damaged (FITC+/PI+) spermatozoa populations were determined in the histogram formed after the analysis. (Figure 2c). In statistical analyses, only the percentage of viable spermatozoa with intact acrosomes (FITC-/PI-) were evaluated (7).

The Ci parameter was determined with Fluo-4 AM (F14201, ThermoFisher). 246 µl of diluted semen was transferred to the microplate wells and 2.5 µl of Fluo-4 AM and 1.5 µl of PI dye were added. In the histogram formed after the analysis, dead spermatozoa (PI+), viable non-capacitated spermatozoa (FLUO-Red), and viable capacitated spermatozoa (FLUO-Green) were determined (Figure 3a). In statistical analyses, only percentage of viable non-capacitated spermatozoa (FLUO-Red) were evaluated (7).

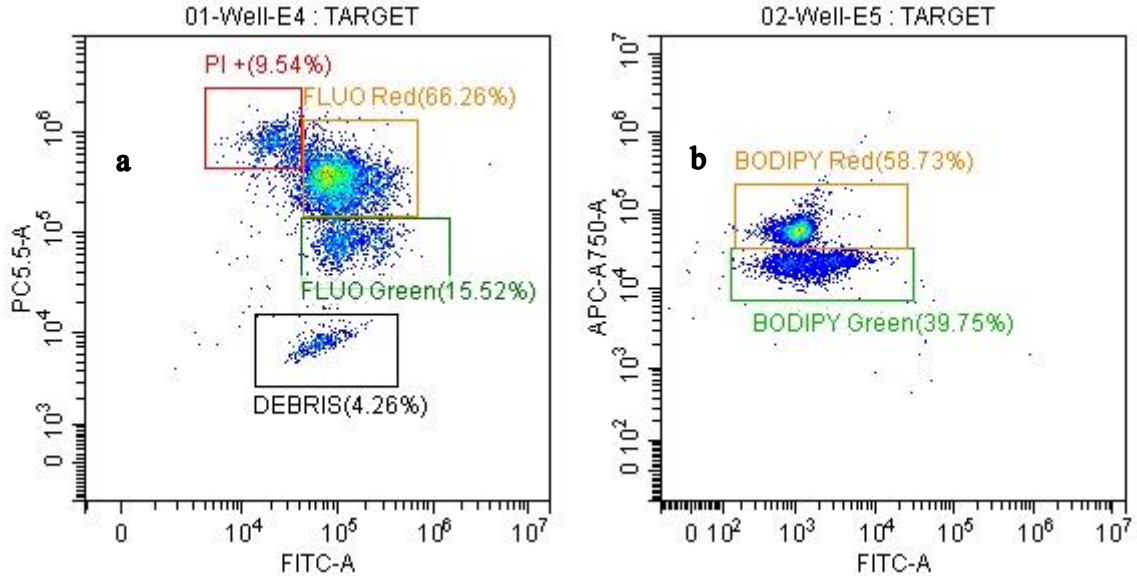

Figure 3: Determination of Ci (a) on FITC vs. PC5.5 and LP (b) on FITC vs.APC-A750 by gating on density plot chart.

Şekil 3: *FITC ve P5.5 düzleminin yoğunluk grafiğine göre Ci (a) ve FITC ve APC-A750 düzleminin yoğunluk grafiğine göre LP (b) analizlerinde kapılama işlemleri.*

The LP analysis was determined by BODIPY-C11 (D3861, ThermoFisher). 247.5 µl of diluted semen were transferred to the microplate wells, and 2.5 µl of BODIPY dye was added. In the histogram formed after the analysis, spermatozoa with a low lipid peroxidation level (BODIPY-Red) and spermatozoa with a high lipid peroxidation level (BODIPY-Green) were determined (Figure 3b). In statistical analyses, only percentage of spermatozoa with a low lipid peroxidation level (BODIPY-Red) were evaluated as % (7).

Before performing the statistical analysis, the data were examined with the Shapiro-Wilk test for normality and the Levene test for homogeneity of variances as parametric test assumptions. Descriptive statistics for each variable were calculated and presented as a table and figure. The Pearson correlation coefficient was used to determine the correlation between spermatological and flowcytometric variables. To test the differences in each parameter between sampling times of the season, General Linear Models with repeated measures design were used. When a significant difference was revealed, any significant terms were compared by Simple effect analysis with Bonferroni adjustment. $P<0.05$ was considered significant in all analyses. Statistical analyses were performed using IBM SPSS Statistics software Version 23.0.

3. Results

According to the results of the statistical analysis, the differences in the Volume, Concentration, TMOT, and PMOT between the BS, MS, and ES periods (Table 1) were statistically insignificant ($p>0.05$).

Table 1: Means of spermatological analysis of the stallion semen according to the BS, MS, and ES periods in the breeding season (Mean ± SE).
Tablo 1: *Aşım sezonu içinde SB, SO ve SS dönemlerine göre aygır spermalarının analiz sonuçlarının ortalamaları (Ort. ± SH)*

Parameters	Periods			Pooled SEM	P-value
	BS	MS	ES		Samp. Time
Volume (ml)	28.833	24.667	24.167	3.642	0.614
Concentration ($x10^6$/ml)	404.021	236.018	273.172	68.586	0.205
TMOT (%)	70.000	70.750	72.083	3.695	0.922
PMOT (%)	32.417	31.000	28.500	3.499	0.727
Viability (%)	19.593 ^{b}	44.340 ^{a}	56.850 ^{a}	4.068	<0.001
HMMP (%)	42.992 ^{b}	57.072 ^{a}	47.202 ^{ab}	3.528	0.023
AI (%)	25.448 ^{b}	36.118 ^{ab}	42.236 ^{a}	4.338	0.031
Ci (%)	52.314 ^{a}	44.970 ^{ab}	40.913 ^{b}	2.457	0.008
LP (%)	56.647 ^{a}	45.130 ^{b}	37.863 ^{b}	2.718	<0.001

^{a,b}: Differences between the means in the same line with different letters are statistically significant ($p < 0.05$).
^{a,b}: Aynı satırdaki farklı harflere ait ortalamalar arasındaki farklılıklar istatistiksel olarak anlamlıdır ($p<0.05$).

In the flowcytometrical evaluations (Table 1; Figure 4), the viability values were determined to be higher in ES and MS than in BS ($p<0.001$), while the best viability results were obtained in the ES period. The highest values of the HMMP were obtained in MS ($p<0.05$). Although the ES values were determined to be numerically lower than MS, they were statistically insignificant. The lowest HMMP values were obtained from semen collected in BS. The results of the AI analysis were found to be lower in the BS period, and reached the highest values in the ES period ($p<0.05$), similar to Viability. Although the values of AI results in MS are numerically lower, they were not statistically significant according to ES values. While the Ci ($p<0.01$) and LP ($p<0.001$) parameters had the highest values in the BS period, unlike the results of other examinations, they showed a gradual decrease until the ES period. Although the MS values of the Ci parameter were lower than the BS, they were concluded to be statistically similar. The measured values of the LP analysis results in the BS period were statistically significant and higher than the other two periods.

4. Discussion and Conclusion

Although it has been reported in many studies that there are differences in stallion semen in breeding and non-breeding seasons, conflicting results are still presented. On the other hand, although it is thought that there may be differences in semen quality in the official breeding season, which covers about six months and three seasons, few studies have been found on this subject (1). In the present study, the differences and similarities observed in the spermatological parameters of Arabian stallions in three different periods, such as BS (15th February – 15th March), MS (15th April – 15th May), and ES (30th May – 30th June), of the official breeding season (15th February – 30th June).

Volume, concentration TMOT and PMOT are considered the basic parameters of a spermatological examination. In particular, these parameters can be easily performed without the necessities of laboratory conditions, as well as the fact that TMOT ($r=0.40$) and PMOT ($r=0.46$) have a weakly positive correlation with fertility. These values form the basis of every spermatological examination, since volume and concentration are used in converting the parameters expressed as percentages into numerical values (11).

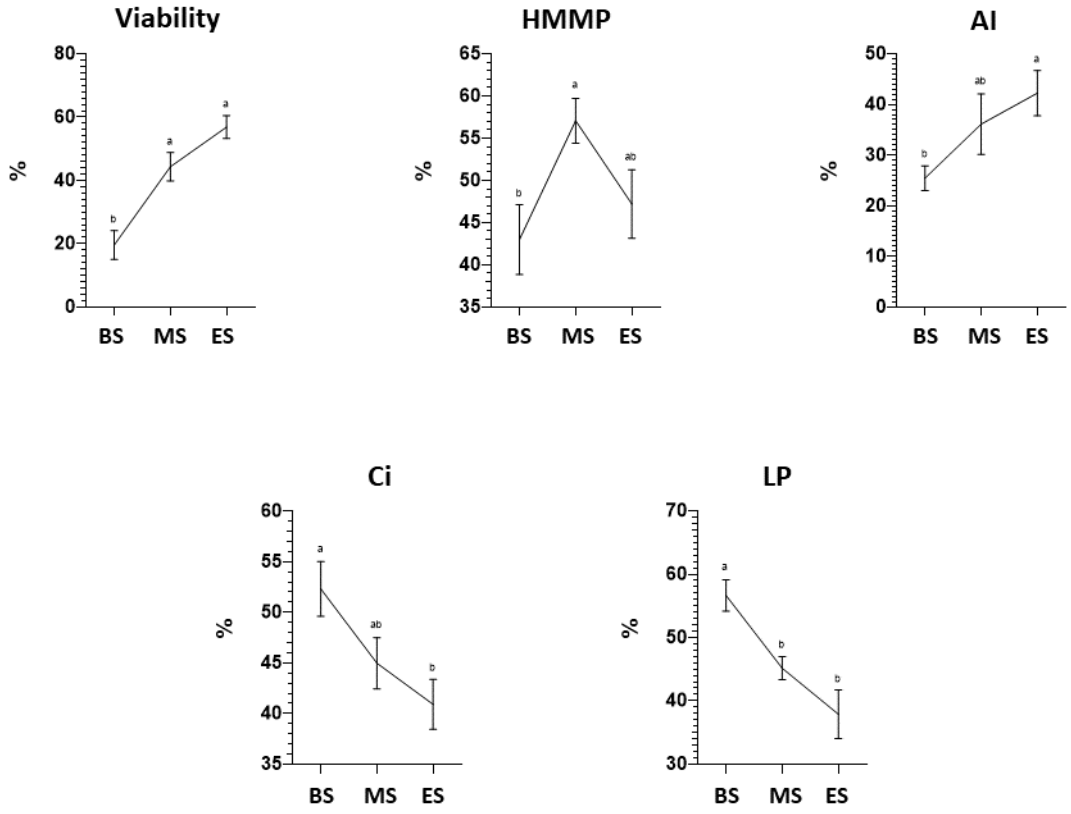

Figure 4: Differentiations in the measurements of flowcytometrical analyses of the stallion semen according to the BS, MS, and ES periods in the breeding season. a,b; Differences between the means in the same graphic with different letters are statistically significant ($p < 0.05$).

Figure 4: *Aşım sezonunda SB, SO ve SS periyotlarına göre aygır spermasının akış sitometri analizleri sonuçlarının değişimleri. a,b; Aynı grafikteki farklı harflere ait ortalamalar arasındaki farklılıklar istatistiksel olarak anlamlıdır (p<0.05).*

In a study, it was reported that the highest volume of semen and the lowest concentration were obtained from semen handled in the summer season (8). While the volume of semen increases, the concentration of spermatozoon decreases in these seasonal variations, causing the total number of spermatozoa in an ejaculate to remain the same. On the other hand, Gamboa et al., (1), concluded that the concentration values were statistically significant and higher in the January-March period compared to the March-May and May-July periods. In a different study on this subject, it was found that while volume and concentration parameters were not reported, the lowest total sperm count was obtained in the winter months (12). Otherwise, it was concluded that the motility values observed in fresh semen samples were at the lowest level in the winter months (4, 8), while they were similar in other seasons and reached the highest numerical values in the summer months. In a study that evaluated the semen collected in December, March, and June, it was mentioned that there was no statistical difference in terms of motility and progressive motility parameters in those months (13). Similar to the present study, Gamboa et al. (1) observed the highest progressive motility in the March-May period (39.24%) and the highest progressive motility in the May-July period (22.75 %). In the present study, unlike other studies, it was concluded that the volume, concentration, TMOT, and PMOT values of semen collected during the season at different periods in Arabian stallions were statistically insignificant. Numerically, the highest TMOT was observed in the ES period, while the highest volume, concentration, and PMOT values were obtained in the BS period. When these data were evaluated, it could be concluded that the findings obtained were mostly different. It can be thought that this situation may occur due to the geographical locations where the studies were conducted, the changes in daylight duration, and the strong differences between the seasons. In addition, the cyclic

pattern of reproductive activities of different breeds of stallions may differ according to the breed characteristics they belong to (12). In the study, it was revealed that the volume, concentration, TMOT, and PMOT parameters of Arabian stallions breeding in Türkiye did not change in a statistically significant way during the season and maintained similar values throughout the breeding season.

In addition to basic spermatological parameters, detailed examination methods that allow the functional characteristics of spermatozoa to be evaluated, thanks to the development of technology and ease of access, have begun to be integrated into the semen examination system. These methods, which were first used in spermatology as fluorescent dyes and subjective evaluation methods, can now be applied in an easy, fast, practical, and objective way with the development of flowcytometry technology. In the present study, the changes in Viability, AI, HMMP, Ci, and LP parameters in Arabian stallion semen during the breeding season were evaluated. Studies have reported that Viability values are higher in the spring than in the summer (8, 12). In addition, Crespo et al., (12) reported a decrease in sperm viability in winter, while Janet et al., (8), on the contrary, reported a decrease in summer. It was concluded that the viability of stallion semen evaluated in different periods during the breeding season was higher between January and May compared to the May-July period (1). On the other hand, the results obtained in the AI evaluation are quite conflicting. In the studies examining the acrosome integrity parameter, Janett et al. (8) obtained the highest AI value in the spring season, while Gamboa et al., (1) obtained the best AI examination result in the semen samples evaluated in the May-July period. On the other hand, Mislei et al. (7) stated that the highest AI and HMMP values were obtained in winter, while Ci and BODIPY values were obtained in summer. It was observed that the Viability and AI parameters were at their lowest levels in BS and increased towards ES in the present study. On the contrary, Ci and BODIPY values have been obtained highest in BS and lowest in ES. While HMMP values have been low in BS and ES, they have shown a significant increase in MS. When the presented study and other studies are evaluated, it could be observed that quite diverse and conflicting results have been obtained. Since similar functional examinations might be performed with many different methods, staining, and measurement procedures, it could be thought that different results can be obtained in this way. Furthermore, it can be seen that the seasons and periods in which the evaluations have been made were concluded different in all studies. In addition, many factors such as breeds, age, care-feeding conditions, past reproductive activity, and frequency of ejaculation during the season, might change semen quality and spermatological parameters. In the present study, the changes in the Viability, AI, HMMP, Ci, and LP parameters of Arabian stallions breeding in Türkiye in different periods within a breeding season were tried to be revealed.

It could be concluded from the results of the present study that basic spermatological parameters are insufficient in determining the quality of semen in stallions and that more detailed flowcytometric examinations should be evaluated. Moreover, when other studies are examined, it could be seen that the semen quality of stallions should be evaluated in terms of geographical location, care-feeding conditions, breed, and age. In conclusion, it has been observed that the best semen quality can be obtained in the period of MS (15 April – 15 May) for young (5-10) years old Arabian stallions breeding in Türkiye. In further studies, evaluations of different age groups, regions, management conditions, breeds, and especially fertility results will allow more precise and accurate results to be obtained.

Conflict of Interest

The authors declared that there is no conflict of interest.

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Authors' Contributions

Motivation / Concept: Kemal Tuna Olğaç
Design: Kemal Tuna Olğaç
Control/Supervision: Mehmet Borga Tırpan
Data Collection and / or Processing: Kemal Tuna Olğaç, Mehmet Borga Tırpan
Analysis and / or Interpretation: Kemal Tuna Olğaç, Mehmet Borga Tırpan
Literature Review: Kemal Tuna Olğaç
Writing the Article: Kemal Tuna Olğaç
Critical Review: Mehmet Borga Tırpan

Ethical Approval

All procedures involving study animals in the experiment were approved by Ankara University Animal Experiments Local Ethics Committee (No:2022-20-178).

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