Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
29 (1): 1-8, 2023
DOI: 10.9775/kvfd.2022.27920

RESEARCH ARTICLE

Determination of Seminal Characteristics in Turkish Aseel Roosters

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Article ID: KVFD-2022-27920 Received: 11.06.2022 Accepted: 15.01.2023 Published Online: 30.01.2023

Abstract: Ornamental poultry is a hobby that has been of interest for centuries. The history of ornamental poultry associations in Europe dates back to the 19th century and to the Ottoman period in Turkiye. One of the most popular ornamental poultry species is Aseel roosters. Aseel roosters are indigenous of Pakistan and India, and they have been bred for competition during the Ottoman period. This study aims to determine the spermatological characteristics of Turkish Aseel roosters. In the study, 10 Aseel roosters were used, and semen was collected by the abdominal massage method twice a week. The sperm motility was estimated by a hot plate phase-contrast microscope under 400´ magnification. The sperm concentration of each ejaculate was determined by hemocytometer and percentages of viable, dead, and abnormal spermatozoa was calculated using eosin-nigrosine staining. Acrosome membrane integrity of rooster spermatozoa were assessed using fluorescein isothiocyanate-conjugated peanut agglutin (FITC-PNA). Spermatozoa membrane functionality was assessed with the hypoosmotic (HOS) test. The spermatological data obtained as a result of the experiment are as follows; ejaculate volume average 308.49±12.14 µL, spermatozoa motility 89.66±0.47%, spermatozoa concentration 2.39±0.10x10^9/mL, The general total morphological defect rate 17.19±0.75%, viability 85.45±0.88%, acrosome integrity rates 98.26±0.09%, and pH 7.81±0.02.

Keywords: *Aseel rooster, Ornamental poultry, Seminal characteristic*

Türk Aseel Horozlarında Seminal Özelliklerin Belirlenmesi

Öz: Süs kümes hayvanları yüzyıllardır ilgi gören bir hobidir. Avrupa'daki süs kanatlı birliklerinin tarihi 19. yüzyıla, Türkiye'de ise Osmanlı dönemine kadar uzanmaktadır. En popular süs kanatlı türlerinden biri Aseel horozlarıdır. Aseel horozları Pakistan ve Hindistan'a özgüdür ve Osmanlı döneminde yarışmalar için yetiştirilmiştir. Bu çalışma, Türk Aseel horozlarının spermatolojik özelliklerini belirlemeyi amaçlamaktadır. Çalışmada 10 adet Aseel horozu kullanıldı ve haftada iki kez karın masajı yöntemiyle sperma alındı. Sperm motilitesi, 400´ büyütme altında ısıtma tablalı faz contrast mikroskobu ile değerlendirildi. Her ejakülatın sperm konsantrasyonu hemositometrik yöntem ile belirlendi ve canlı, ölü ve anormal sperm yüzdeleri eozin-nigrosin boyaması kullanılarak hesaplandı. Spermatozoaların akrozom membrane bütünlüğü, floresein izotiyosiyanat konjuge peanut aglutin (FITC-PNA) kullanılarak değerlendirildi. Spermatozoa membrane işlevselliği hipoozmotik (HOS) testi ile değerlendirildi. Deney sonucunda elde edilen spermatolojik verilere göre ejakülat hacmi ortalaması 308.49±12.14 µL, spermatozoa motilitesi %89.66±0.47, sperm konsantrasyonu 2.39±0.10x10^9/mL, toplam morfolojik bozukluk oranı %17.19±0.75, canlılık %85.45±0.88, akrozom bütünlük oranları %98.26±0.09 ve pH 7.81±0.02 olarak bulunmuştur.

Anahtar sözcükler: *Aseel horozu, Spermatolojik özellikler, Süs kümes hayvanları*

How to cite this article?

Günay E, Arıcı R, Şenlikçi H, Yağcıoğlu S, Eser A, Demir K, Alkan S, Çeşmeci M: Determination of seminal characteristics in Turkish aseel roosters. *Kafkas Univ Vet Fak Derg,* 29 (1): 1-8, 2023.
DOI: 10.9775/kvfd.2022.27920

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Introduction

Aseel roosters have been famous for their well-developed musculature, body formation, pugnacity, stamina and fight in Turkiye and all over the world. Aseel roosters are indigenous of Pakistan and India, and they have been bred for competition during the Ottoman period [1-3]. They are also preferred for their immunity, adaptation to harsh conditions and organic meat production [4,5]. The history of ornamental poultry associations in Europe dates back to the 19th century and to the Ottoman period in Turkey. However, organized and more scientific breeding started in Türkiye in 2016 [6]. Aseel roosters are one of the most popular ornamental poultry breeds in Turkiye. Producing the most valuable breeders is the common goal of all producers. However, this breed suffers from low egg production, poor hatching rates, and low fertilization [7]. Also, the quality of semen directly effects the fertility level. To get rid of the reproductive problems, it is necessary to select the Aseel individual with the best spermatological characteristics and high breeding value. Fertility in roosters depends on quality and quantity of semen, as well as the mating capacity [8-10]. Sperm fertilization is directly related to the ultrastructure of spermatozoa, motility, morphology, concentration, DNA fragmentation and genetic composition [11-15]. Semen quality in roosters is analyzed by using parameters such as motility, viability, membrane, and acrosome integrity [10,16].

It was aimed in this study to reveal the spermatological characteristics of Turkish Aseel roosters. Thus, reference values were obtained for the determination of quality breeders, cooling, and freezing of semen, and artificial insemination in Aseel roosters. These values were determined for motility, volume, concentration, pH, membrane functionality, acrosome integrity, morphological defects and viability.

Materials and Methods

Ethical Approval

The study was conducted with the permission of Istanbul University-Cerrahpasa Animal Experiments Local Ethics Committee (IUCHADYEK) with the approval number 2022/41

Animals and Semen Collection

The study was carried out at Istanbul University-Cerrahpasa Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination. Ten Assel breed roosters were used in the study at 2 years, weighing 2.1 to 3.4 kg with the phenotypic characteristics of the Aseel breed [3].

The roosters were kept separately (length C width C height) in individual cages measuring 1 m x 75 cm x 75 cm. and fed *ad libitum* with commercial chicken food. The roosters were trained in semen collection by abdominal massage. Semen was collected by abdominal massage twice a week for 5 weeks [17]. Semen samples were taken individually into 1.5 mL microcentrifuge tubes and then transported to the laboratory within 5 min.

Macroscopic Evaluation of Semen

The appearance of semen samples was scored by visual inspection on a scale of 1 to 5 [18]. Watery or clear semen was given 1 point, white smoky semen 2 points, medium white semen 3 points, dark, white semen 4 points, and very viscous, chalky white semen samples 5 points. The volume was measured with an automatic pipette and pH with pH strips without dilution [19,20].

Sperm Mass Activity

The sperm mass activity was estimated by a hot plate phase-contrast microscope under 10x magnification [21].

Sperm Motility Analysis

The sperm motility was estimated by a hot plate phase-contrast microscope under 400x magnification. Motility was expressed as the percentage of motile spermatozoa with moderate to rapid progressive movement. At least 3 microscopic fields were examined for each sample.

Sperm Concentration

The sperm concentration of each ejaculate was determined by hemocytometer in Formol (1%) saline solution ata1:400 ratio and expressed as billion (10^9) per mL [22].

Sperm Viability and Morphology

Percentages of viable, dead, and abnormal spermatozoa were calculated using eosin-nigrosine staining [23]. One drop of semen was placed on a clean, warm glass slide and mixed with a mixture of 1 drop of 5% eosin and 2 drops of 10% nigrosine stain. The slides were prepared and air-dried. One hundred spermatozoa in each preparation were examined under a fluorescent microscope (400´ magnification). Both fully and partially stained spermatozoa were counted as dead *(Fig. 1)*. Visible abnormalities in the head, neck, mid-piece and tail regions were used to estimate the percentage of abnormal spermatozoa by counting a total of 100 spermatozoa. *(Fig. 2, Fig. 3, Fig. 4)*.

Acrosome Integrity

Acrosome membrane integrity of rooster spermatozoa was assessed using fluorescein isothiocyanate-conjugated peanut agglutin (FITC-PNA). Principally, FITC-PNA labels the acrosome region of acrosome-reacted spermatozoa. The stock solution of FITC-PNA (1 mg/mL) was diluted (1:10) in PBS before staining. From all animals, 5 µL of

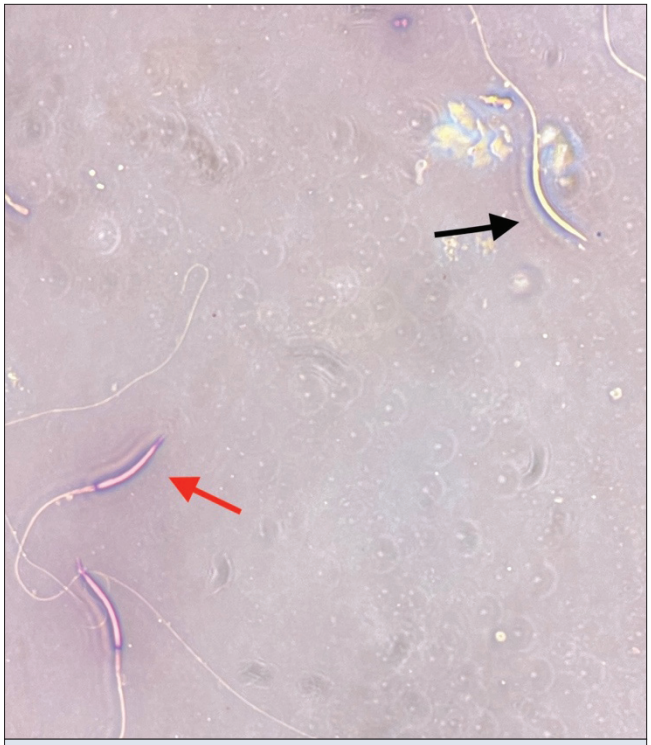

Fig 1. Live *(black arrow)* and dead *(red arrow)* spermatozoa

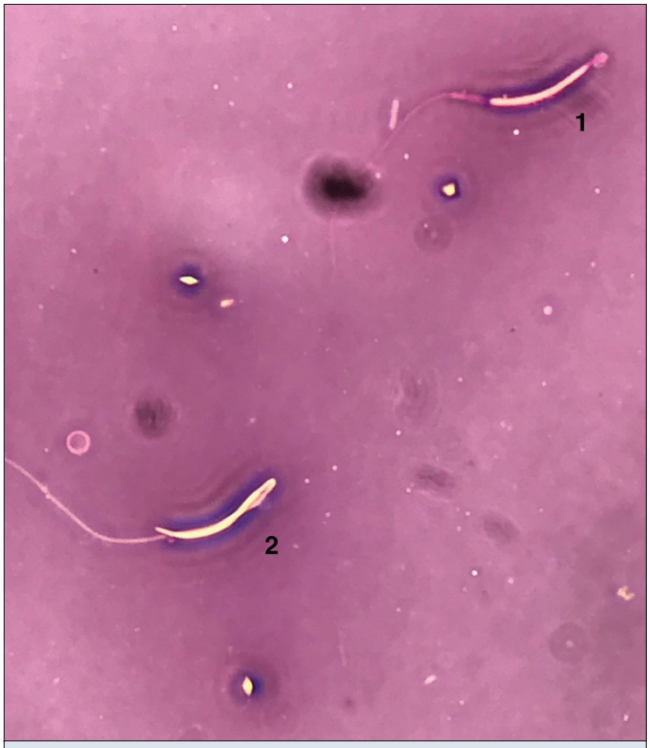

Fig 3. Acrosome swelling (1) and crooked beck (2) spermatazoa

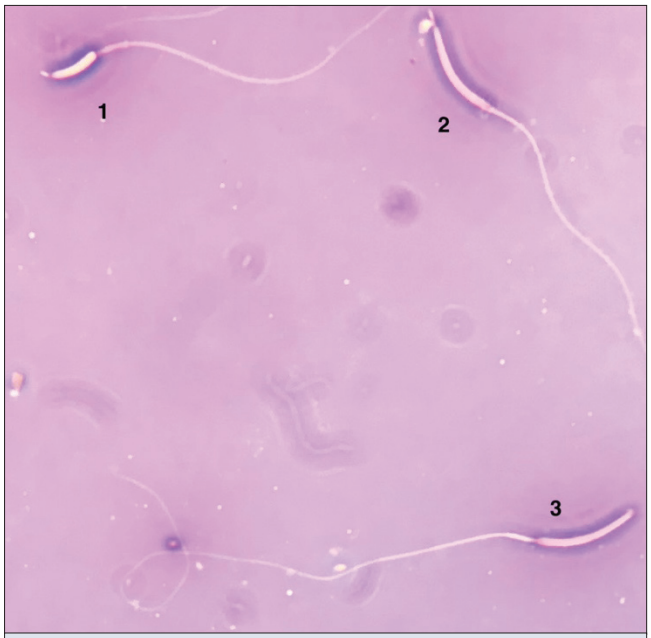

Fig 2. Small head (1), normal (2) and without acrosome (3) spermatozoa

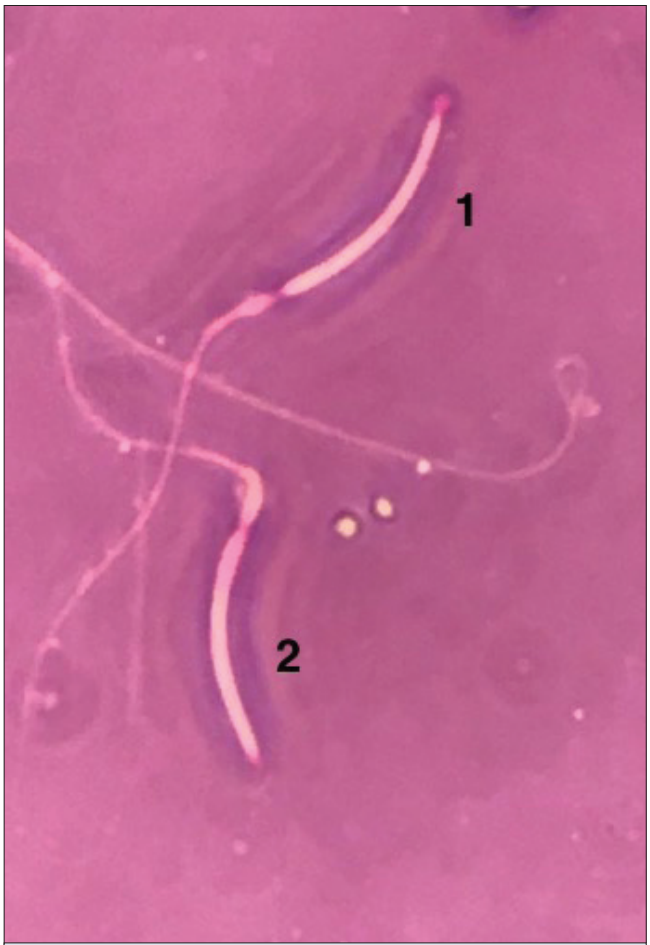

Fig 4. Acrosome swelling (1) and acrosome detached (2) spermatazoa

semen samples were diluted with 295 µL Tris-buffered media and 5 µL of FITC-PNA working solution were added to the diluted semen. Samples were loaded into a 96-well plate and incubated for 10 minutes at room temperature. Measurements were performed on a Guava® easyCyte™ (Luminex) flow cytometer using Guava® InCyte™ software. FITC-PNA positive (560 nm emission wavelength) and

negative spermatozoa were detected. Per sample, 10.000 events were acquired and gathered in diagrams *(Fig. 5)*.

Spermatozoa Membrane Functionality

Spermatozoa membrane functionality was assessed with the hypoosmotic (HOS) test, as described by Zhanget al.[24]. For the membrane test, 25 µL ofthe semen sample and 975 µL of HOST (100 mOsm /kg, 57.6 mM fructose and 19.2 mM sodium citrate) solution were mixed in Eppendorf tubes and incubated at 37°C for 30 min. 5 µL of the incubated solution was dropped onto a slide (37°C) and covered with a coverslip. 200 spermatozoa were counted under a phase contrast microscope at 100´ magnification with immersion oil. Sperm with a curved tail, swollen head, and spiral-like appearance were considered HOST test positive.

Statistical Analysis

Statistical evaluation of semen pH, viability, mass activity and HOST results was carried out by the "Kruskal-Wallis Test". "One-way Analysis of Variance" (ANOVA), followed by "Duncan's Multiple Range Test" was used to evaluate the data obtained from semen volume, concentration, subjective motility, total morphological defects and acrosomal integrity examinations. In addition, bivariate correlations between the semen variables (pH, semen volume, semen color, mass activity, subjective motility, viability, concentration, HOST+, total morphological defects and acrosomal integrity) were assessed by "Spearman's correlation coefficients *(r_s)*. Statistical analyzes were performed using the SPSS Version 22.0 for Windows (SPSS Inc., Chicago, IL, USA). The results were represented as mean ± standard error. Differences with values of P<0.05 were regarded as statistically significant.

Results

Average pH, volume and color scale results of semen obtained from 10 roosters are presented in *Table 1*. Semen pH values were similar in all roosters (C) (P>0.05). The general average sperm pH was 7.81±0.02. The lowest and the highest mean ejaculate volume were 188.46±24.95 µL and 469.64±42.66 µL respectively, and semen volume differed between individuals (P<0.05). It was determined that the average semen volume of 10 roosters was 308.49±12.14 µL Similarly, it was determined that the color of semen classified by the scale method between + and ++++, differed individually (P<0.05).

Microscopic examination results and acrosome integrity rates determined by flow cytometry are shown in *Table 2*. Mass activity (3.85±0.04), viability (85.45±0.88%), HOST positive (94.25±0.25%), and acrosome integrity rates (98.26±0.09%), were similar in all roosters (P>0.05). The lowest percentage of subjective motility was determined in the C7 rooster as 82.77±4.41%. The highest motility percentages were found in the cocks C1, C2 and C9, and

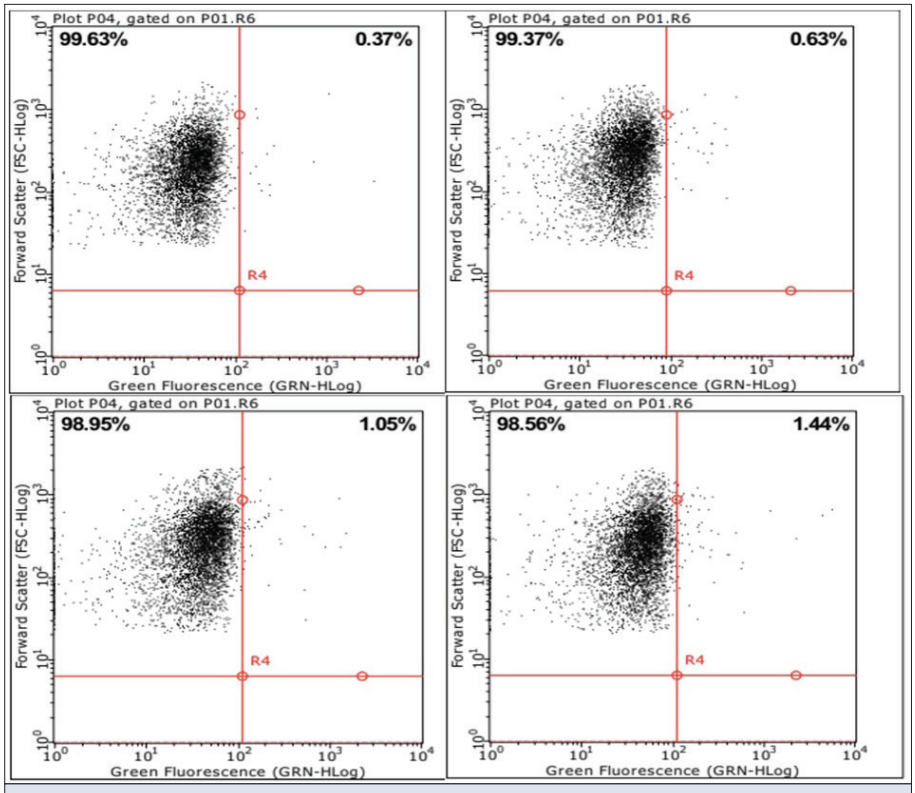

Fig 5. Acrosome integrity images in individual semen samples

Table 1. Some spermatological characteristics of Aseel cocks

Cock No	pH	Volume (µL)	Semen Color (1-5)
	$\bar{x}\pm S_{\bar{x}}$	$\bar{x}\pm S_{\bar{x}}$	$\bar{x}\pm S_{\bar{x}}$
C1	7.84±0.06	214.64±20.62^{cd}	2.71±0.24^{a}
C2	7.80±0.07	306.42±36.03^{bc}	3.00±0.25^{a}
C3	7.69±0.12	188.46±24.95^{d}	2.53±0.18^{ab}
C4	7.85±0.06	260.41±23.31^{cd}	2.33±0.25^{ab}
C5	7.88±0.06	301.15±25.91^{bc}	2.53±0.24^{ab}
C6	7.92±0.04	469.64±42.66^{a}	2.76±0.20^{a}
C7	7.72±0.18	253.63±22.77^{cd}	1.90±0.21^{b}
C8	7.88±0.06	300.76±31.71^{bc}	2.53±0.24^{ab}
C9	7.83±0.07	383.33±25.68^{ab}	2.75±0.13^{a}
C10	7.65±0.13	421.00±43.82^{a}	2.80±0.24^{a}
General	7.81±0.02 (*n*:125)	308.49±12.14 (*n*:126)	2.60±0.07 (*n*:125)

^{abc} *Values without common superscripts in the same column are statistically different, P<0.05*

the motility percentages of these cocks were found to be higher than the cocks C3 and C7 (P<0.05). The general average of subjective motility was 89.66±0.47% (*n*: 119). Spermatozoa concentrations of C6 and C10 were higher in cocks compared to C3, C4, and C7 (P<0.05). The overall spermatozoon concentration was 2.39±0.10 ´ 10^9/mL. The total morphological defect rates in fresh rooster semen also differed individually (P<0.05). The general total morphological defect rate 17.19±0.75% (*n*:120) (Fig.5). With the lowest in C8, and the highest in C3 and C7. Also, the results show the correlation between spermatological examinations performed on fresh semen of Aseel roosters.

Discussion

Artificial insemination has a key role in overcoming reproductive problems. One of the most important factors of artificial insemination is the use of high fertility breeder roosters in industrial and ornamental poultry. Especially in ornamental poultry, it is imperative to determine the spermatological characteristics in selecting high-quality breeders and increase the success of artificial insemination.

In the present study, the lowest and highest seminal volume of total 125 ejaculates were 0.18846±0.02495 mL and 0.46964±0.04266 mL respectively with a mean volume of 0, 30849±12.14 mL. This result was similar to the results of Mavi et al.[10] (0.36±0.08) and lower than Keskin et al.[25]. The seminal volume for Leghorn and Gerze roosters has been reported almost similar to the result of the present study [10,26-28]. It is noteworthy that the mean seminal volume is reported as 0.7±0.01 mL in Denizli Roosters [27,28] which can be attributed to racial differences, nutrition, environmental factors, and hormone level differences.

Mavi et al.[10] found the volume in Aseel roosters. Alkan et al.[29] and Keskin et al.[25] found a mean ejaculate volume 0.27 mL and 0.6±0.1 mL for Erbro roosters, respectively. Chalov [26] 0.3 mL for Leghorn roosters and Tuncer et al.[27] 0.70±0.01 mL found for Denizli roosters and 0.37±0.006 mL for Gerze roosters. In this study similar results were obtained with Mavi et al.[10]. The different results obtained in other studies may be due to breed differences. However, various factors such as semen collection frequency, stress, nutrition, individual and species difference can affect the seminal volume.

Table 2. Average rate of mass activity, subjective motility, viability, concentration, HOST +, total morphological defects and acrosomal integrity in Aseel cocks

Cock No	Mass Activity (+, +++++)	Subjective Motility (%)	Viability (%)	Concentration (x10^9/mL)	HOST + (%)	Total Morphological Defects (%)	Acrosomal Integrity (%)
	$\bar{x}\pm S_{\bar{x}}$	$\bar{x}\pm S_{\bar{x}}$	$\bar{x}\pm S_{\bar{x}}$	$\bar{x}\pm S_{\bar{x}}$	$\bar{x}\pm S_{\bar{x}}$	$\bar{x}\pm S_{\bar{x}}$	$\bar{x}\pm S_{\bar{x}}$
C1	3.92±0.07	91.15±1.15^{a}	83.78±3.27	2.57±0.38^{abc}	94.50±0.64	19.07±1.80^{ab}	98.26±0.23
C2	3.92±0.07	91.78±0.66^{a}	83.21±3.00	2.48±0.21^{abc}	93.60±0.83	19.14±2.26^{ab}	98.06±0.37
C3	3.76±0.16	86.66±1.12^{b}	90.38±0.87	1.72±0.28^{cd}	94.73±0.51	20.84±3.00^{b}	98.29±0.24
C4	3.83±0.20	90.45±0.81^{ab}	86.09±1.93	1.90±0.27^{bcd}	94.25±0.57	15.09±2.03^{ab}	98.12±0.36
C5	3.84±0.10	90.83±0.83^{ab}	86.76±2.67	2.61±0.28^{abc}	94.23±1.07	17.07±2.56^{ab}	98.13±0.31
C6	4.00±0.00	90.71±0.71^{ab}	81.76±4.04	3.07±0.32^{a}	95.23±0.70	13.92±1.60^{ab}	98.59±0.23
C7	3.20±0.35	82.77±4.41^{c}	85.20±2.79	1.22±0.22^{d}	91.05±1.48	28.00±2.49^{c}	98.41±0.27
C8	3.92±0.07	89.23±1.11^{ab}	89.30±2.87	2.45±0.26^{abc}	95.11±0.57	12.41±1.59^{a}	98.50±0.31
C9	4.00±0.00	91.25±0.65^{a}	82.66±1.93	2.72±0.39^{ab}	94.50±0.65	14.18±1.75^{ab}	98.02±0.39
C10	4.00±0.00	89.44±1.00^{ab}	85.50±2.17	3.08±0.36^{a}	94.75±0.38	14.50±1.93^{ab}	98.28±0.31
General	3.85±0.04 (n: 125)	89.66±0.47 (n: 119)	85.45±0.88 (n: 123)	2.39±0.10 (n: 126)	94.25±0.25 (n: 123)	17.19±0.75 (n: 120)	98.26±0.09 (n: 125)

^{abc} *Values without common superscripts in the same column are statistically different, P<0.05*

Table 3.*Correlation values between spermatological examinations*

Parameters	Correlation	Statistical Data			Spermatological Examinations									
		n	$\bar{x}$	$S_{\bar{x}}$	SC	pH	VOL	MA	MOT	CON	VIA	HOST	AI	TMD
Spermatological examinations	SC	125	2.60	0.07	-									
	pH	125	7.81	0.02	.080	-								
	VOL	126	308.49	12.14	.210*	.187*	-							
	MA	125	3.85	0.04	.214*	.085	.220*	-						
	MOT	119	89.66	0.47	.268**	.099	.256**	.406**	-					
	CON	126	2.39	0.10	.471**	.101	.372**	.280**	.331**	-				
	VIA	123	85.45	0.88	.133	.198*	.018	.043	.020	.220*	-			
	HOST	123	94.25	0.25	.444**	.227*	.219*	.200**	.193*	.367**	.256*	-		
	AI	125	98.26	0,09	-.078	-.020	.070	.077	-.077	-.091	-.100	.091	-	
	TMD	120	17.19	0.75	-.099	-.069	-.123	-.228*	-.082	-.217*	-.102	-.005	-.192*	-

SC: *Semen color,* ***VOL:*** *Volume,* ***MA:*** *Mass activity,* ***MOT:*** *Motility,* ***CON:*** *Concentration,* ***VIA:*** *Viability,* ***HOST:*** *Hypo-osmotic swelling test (+),* ***AI:*** *Acrosomal integrity,* ***TDM:*** *Total morphological defects, * P<0.05, ** P<0.01*

The color of semen was evaluated from 1 to 5 points from watery to creamy, with an average value of 2.60±0.07. According to Mavi et al.[10] color of Aseel roosters semen is creamy. In our study, color had an average score of 4 and more and, a high correlation was observed between semen color and semen concentration (P<0.01). Particular attention should be paid to this feature in the selection of breeders.

Spermatozoa motility is one of the most reliable parameters and gives information about the fertilization ability of semen. The higher is motility the better the fertilization results [30]. Alkan et al.[29] and Keskin et al.[25] determined spermatozoa motility as 85.83% and 79.4±11.5%, respectively. Tuncer et al.[27] and Tuncer et al.[28] determined spermatozoa motility 72.32±0.80% for Denizli roosters and 74.28±0.73% for Gerze roosters. In this study, the mean spermatozoa motility was 89.66±0.47%. which is higher than the values of several studies [25,27-29]. Especially, the motility result of the present study is higher than the result of another study with Aseel breed [10].

For Aseel roosters Mavi et al.[10] determined a spermatozoa motility of 75.87±5.73%, whilst, in our study, the mean overall sperm motility was 89.66±0.47%. The difference between the results of two studies with the same breed can be attributed to differences in nutrition, accommodation, and climatic differences.

Spermatozoa concentration directly affects the number of spermatozoa in sperm storage tubes and plays an important role in fertilization [31]. The mean spermatozoa concentration was 2.39±0.10 x10^9/mL in this study. The concentration of spermatozoa is affected by factors like individuality, breed, age, and season. Siudzinska and Lukaszewicz [32] reported that the mean sperm concentration in White Crested Black Polish cocks was 4.7 x 10^9/mL and in Black Minorcas breeds 4.2 x 10^9/mL. On the other hand, Tuncer et al. reported 2.4 x 10^9/mL and 2.38±0.03x10^9/mL in Gerze and Denizli roosters [27,28]. In the present study, a positive correlation was found between semen volume and concentration (P<0.01). It is recommended to consider these values when choosing a breeder.

Seminal pH has been associated with metabolic rate and spermatozoa motility. Turkey and rooster semen can tolerate a pH range of 6-8 [33], and the pH was found to be 7.81±0.02 *(n*: 125) in this study. Tuncer et al.[27] and Tuncer et al.[28] reported pH as 7.71 in Gerze roosters and 7.68±0.01in Denizli roosters. The results of these studies are all in the tolerance range for rooster semen.

Fertilization is affected more by morphological defects of spermatozoa than motility [19,33]. Many researchers state that acrosome defects of rooster semen are the most important effective trait of fertility [19]. In this study, total morphological defects *(Fig. 2, Fig. 3, Fig. 4)* were determined as 17.19±0.75% and total acrosome integrity *(Fig. 1)* as 98.26±0.09%. The rate of viable spermatozoa was 85.45%±0.88% and the total HOST was 94.25±0.25%. This study had resembling data to from other researchers [25,29,34,35]. However, Mavi et al.[10] achieved lower motility 75.50±7.99% and vitality 77.20±5.81% results. Various results of spermatological characteristics in other studies may be due to season, temperature, photoperiod, age, breed, ejaculation frequency, and individual differences. Testosterone has an important role in the formation and display of secondary sex characteristics and affects semen quality and production. High testosterone levels may explain the bellicose temperament

and original phenotype of Aseel roosters. Based on this, the relationship between testosterone levels and spermatological characteristics in Aseel roosters may be investigated.

This study can be considered the very preliminary study on the Turkish Aseel rooster's semen and aims to determine the spermatological characteristics of the species. The results will contribute to further research on Aseel cocks and artificial insemination with fresh, chilled, or frozen semen. It also provides up-to-date data for breeders of this interesting bird species.

Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author (E. Günay). The data are not publicly available due to privacy or ethical restrictions.

Ethical Approval

The study was conducted with the permission of Istanbul University-Cerrahpasa Animal Experiments Local Ethics Committee (IUCHADYEK) with the approval number 2022/41

Funding Support

This study was supported by Istanbul University-Cerrahpaşa BAP. Project ID: 35772. Project code: TSA-2021-35772

Conflict of Interest Statement

The authors declare that there is no conflict of interest in publishing this article.

Author Contributions

SA and KD conceived and supervised this study. EG, RA and HŞ completed the main experimental content. RA and AE collected and analyzed data. EG wrote the first draft of the manuscript. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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