Original Article

Stepwise regression analysis to assess various factors affecting on fresh sperm quality in Ouled Djellal ram

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Abstract

The aim of this study was to evaluate the effect of various factors on fresh semen quality in Ouled Djellal rams. Specifically, we examined the impact of semen collection method, season, ram age, testosterone levels, body weight , and testicular weight. Twelve mature Ouled Djellal rams were used in the trial. Six rams were trained for semen collection using an artificial vagina (AV), while semen from the other six rams was collected using an electroejaculator (EE) biweekly throughout the year. Stepwise regression analysis showed that 62.50% of the variation in sperm volume, 48.60% of the variation in mass motility, 62.70% of the variation in sperm concentration, and 44.20% of the variation in total sperm count can be attributed to the collection method. Overall, significantly better semen quality was recorded for the artificial vagina (AV) collection method compared to the electroejaculator (EE) method. Of the seven variables studied, two factors—method of collection and season—had a significant effect on pH and the percentage of live sperm, accounting for 41.90% and 37% of the variation, respectively. Season was the most important predictor for the percentage of abnormal sperm, characterized by the equation PSA = 0.532 + 1.058 × season, with a correlation coefficient (R) of 0.459. In conclusion, Ouled Djellal rams responded better to AV than to EE for semen collection. Both the collection method and the season were significant factors influencing semen characteristics and can be used as key indicators for selecting superior breeding rams to enhance herd productivity.

Keywords: artificial vagina, electroejaculator, Ouled Djellal ram, sperm, stepwise regression model

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Introduction

The selection of breeding rams is crucial for achieving optimal herd productivity. A selected ram must possess high genetic potential, demonstrate strong libido, and consistently produce quality semen during routine collections. The success of AI or cryopreservation depends on the quality of the gametes, as collected spermatozoa or oocytes will undergo several manipulations before being inseminated back into the female (Chen *et al*., 2020). For optimal semen quality, all physiological processes, including the development of the reproductive system from birth to puberty, spermatogenesis, ejaculation, and mating behavior, must be well-coordinated (Belkhiri *et al*., 2021). Parameters commonly used to assess semen quality include sperm concentration, semen volume, sperm motility, and morphology. Such evaluations help detect and eliminate clear cases of male infertility or subfertility, ensuring that only the most fertile rams are used for breeding purposes (Ihukwumere and Okere, 1990).

Various factors that affect ejaculate quantity and semen quality have been documented in studies. These include method of semen collection season of the year, age, body weight of animal, testicular size, hormonal influence, breed differences, geographical location, temperature, nutritional status of the animal, individual variation, reproductive management, transportation, skill of the semen collector, frequency of semen harvest, responsiveness of the ram, testing methodology, general health of the ram and exposure to chemical agents or radiation (Ihukwumere and Okere, 1990; Zamiri *et al*., 2010; Belkhiri *et al*., 2020 Palacin-Martinez *et al*., 2022).

For domestic small ruminants, semen samples can be collected using one of the following methods: artificial vagina (AV) or electro-ejaculator (EE). Semen collection via AV is widely used because sperm samples obtained by this method are similar to those collected through natural service (Wulster-Radcliffe *et al*., 2001). However, it typically requires a preliminary training period and the use of a dummy or mount ewe. Additionally, limitations such as disease transmission, lameness in rams, and the need for trained rams can affect the use of AV (Barth and Waldner 2002). Electroejaculation (EE) is an alternative semen collection method for species or breeds raised in extensive systems, necessitating different strategies beyond routine reproductive techniques (Pilar Jiménez-Rabadán *et al*., 2013). Additionally, EE can be used repeatedly to collect ejaculates from multiple rams in large numbers without causing harm (Marco-Jiménez *et al*., 2005).

Photoperiod is the primary environmental factor affecting sheep reproduction. While males are not as strongly influenced by photoperiod as females, seasonal changes in semen characteristics and libido have been reported in several breeds (Kridli *et al*., 2007). In rams, a decrease in daylight length increases the rate of spermatogenesis due to melatonin production, which stimulates the release of gonadotropin-releasing hormones (GnRH). Ritar (1993) demonstrated significant seasonal variations in semen quality of small ruminants residing at high or mid-level altitudes. Consequently, many sheep breeds in subtropical areas exhibit seasonal variations in sexual activity similar to those in temperate zones, differing primarily in the duration of sexual activity expression due to the amplitude of photoperiod variation. Despite these changes, males continue to produce fertile spermatozoa and exhibit sexual behavior throughout the year (Kridli *et al*., 2007).

Age is a major contributing factor to differences in semen characteristics. Several studies have investigated the influence of male age on sperm characteristics in rams (Tabbaa *et al*., 2006; Chella *et al*., 2017). These studies have shown that advancing age is associated with a decrease in semen volume and sperm concentration concentration (Ntemka *et al*., 2019). Aging is linked to reduced semen volume due to diminished function of the seminal vesicles and accessory glands (Abah *et al*., 2023).

Androgens play a crucial role in the endocrine microenvironment of sperm maturation as they pass through the seminiferous tubules, rete testis, and efferent ducts (Turner *et al*., 1984). Intratesticular testosterone is essential for initiating and maintaining spermatogenesis, acting indirectly via Sertoli cells. Testosterone diffuses as a paracrine factor into the seminiferous tubules, where Sertoli cells reside, supporting the final phases of spermatogenesis by regulating the secretion of proteins from these somatic testicular cells (Guardo *et al*., 2020).

Sperm output in rams is directly proportional to testicular size, with rams possessing large, symmetrical testes free from defects and being more likely to produce high-quality semen (Oyeyemi *et al*., 2009). Additionally, Body weight also provides information about the physical and physiological maturity of the animal, its semen output, and the birth weight of its offspring (Gouletsou and Fthenakis, 2010).

Currently, there is a lack of information regarding the selection of breeding rams based on semen quality in our country. Few studies in the literature compare semen collection methods in rams. To our knowledge, no research has been conducted that uses stepwise regression analysis to identify the factors influencing sperm parameters. This makes our study one of the first to apply this statistical method to understand the determinants of sperm quality in rams. The objective of this work is to investigate the effects of collection method, season, ram's age, testosterone levels, body weight, and testicular weight on sperm quality. To achieve this, we employed stepwise analyses to determine which factors have the greatest impact on sperm quality. This evaluation could provide new strategies for selecting the best Ouled Djellal breeding rams by assessing semen quality for future semen production and artificial insemination programs.

Materials and Methods

Animal and location: The experiment was carried out in Ouled Djellal province in Algeria, which is located between 34° 25′ 44″ North latitude and 5° 03′ 51″ East longitude and at an altitude range from 230 m mean sea level. The climate is dry and hot in summer (with temperatures between 35 and 45°C during the day and

between 25 and 35 °C at night) and dry and cold in winter (with temperatures between 10 and 20 °C during the day and between -2 and 5 °C at night).
Twelve mature Ouled Djellal rams, aged between 2 and 4 years and weighing between 87 and 104 kg, were raised under a natural photoperiod and included in the study. Each ram was fed a daily diet of 2 kg of hay and 0.8 to 1 kg of commercial concentrate. All rams had free access to water and mineral blocks. Additionally, each ram was ear-tagged, vaccinated against certain infectious diseases, and received multivitamins along with prophylactic doses of anthelmintics prior to the commencement of the experiment.

Semen collection methods: For a total observation period of 12 months from June to July. Twenty four ejaculates per ram were obtained during the breeding season equivalent to short photoperiods (September to February) and non- breeding season equivalent to long photoperiods (March to August), on a biweekly basis. In the Ouled Djellal region, the breeding season has the shortest day length (10.52 hours), while the non-breeding season has the longest day length (15.22 hours).

The rams were randomly allocated to the two treatment groups. The rams were grouped according to method of collecting into two batches: each one of them included six rams (06), with three rams of two years and three rams of four years. Semen of the Ouled Djellal rams in the first treatment group (06) was collected with the aid of the artificial vagina (AV) after training. Semen of the remaining rams (n = 6) was collected with the aid of the electro-ejaculator (EE) (second treatment group) for small ruminants (Minitube, Germany).

Semen evaluation: Immediately after semen collection, the following parameters were evaluated macroscopically: semen volume and semen pH and microscopically for semen wave motion (mass motility), sperm concentration, Spermatozoa total number, sperm viability (percentage live) and sperm morphology (abnormalities).
The volume (V) (mL) of the ejaculates was measured in a conical graduated tube. Universal pH paper was used to approximate the pH of the semen. A drop of pure and undiluted semen was placed in the middle of the pH paper and the color resulting from it matched to the standard colors. To evaluate sperm mass motility (MM) (wave motion), a 20 µL drop of semen was placed on a prewarmed slide (37°C) without a coverslip and examined under a light microscope at 100x magnification. Mass motility was scored as follows: 1 = no perceptible motion, 2 = weak motion without forming any waves, 3 = small, slow-moving waves, 4 = vigorous movement with moderately rapid waves and eddies, and 5 = dense, very rapidly moving waves and eddies. Sperm concentration (C) (×109/mL) was calculated using a spectrophotometer. The spermatozoa total number (NTS) (×109/ejaculat) was calculated with the volume and concentration (volume × concentration). Semen wave motion: 10 µL semen was drawn up in an automatic pipette and placed on a pre-warmed microscope slide (32 °C) and evaluated under a microscope (x10 magnification). This semen wave motion was microscopically scored on a scale of 0 to 5. The percentage of live sperm (PSL) (%) was calculated by dividing the number of live sperm (unstained by eosin-nigrosin) over 200 spermatozoa, counted under a light microscope at 1000 magnification with oil immersion. The percentage of abnormal sperm (PSA) (%) was determined from the same slide.

Physical measurements and blood samples: Body weight, testicular weight, and serum testosterone concentration were recorded biweekly. Body weight (BW) was measured using an electronic weighing scale, while testicular weight (TW) was determined volumetrically using the Archimedes principle of water displacement in a measuring cylinder.
Jugular blood samples were collected at 08:00 h from each ram into dry tubes and immediately centrifuged at 1500 × g for 10 minutes. The harvested plasma was stored at –20 ^{o}C until hormone analysis. Plasma testosterone concentration was measured in all samples using an electro-chemiluminescence immunoassay (ECLIA) (Belkhiri *et al*., 2020)

Statistical Analysis: Statistical analysis was performed using SPSS 20.0 (SPSS Inc, Chicago, IL, USA) and results were expressed as the mean ± standard error of the mean (SEM). The Kolmogorov-Smirnov test was used to assess the normality of data distribution. Differences between collection methods (AV vs. EE), season (breeding vs. non-breeding), ram's age (2 years vs. 4 years), testosterone levels (≤5 ng/ml vs. >5 ng/ml), body weight (>90 kg vs. ≤90 kg), and testicular weight (>800 g vs. ≤800 g) were analyzed using Fisher's exact test where appropriate. Body weight, testicular weight, and testosterone level data were categorized into groups based on their median values.

Stepwise regression is a procedure in which predictor variables are investigated sequentially; variables are entered into or moved from the initial model one by one (Mundry and Nunn, 2009). The evaluation should include factors that can currently explain changes in sperm quality, so the results of this analysis can provide practical, feasible, and effective recommendations for improving ram sperm production. Based on available data, this paper examines six factors (collection method, season, age, testosterone levels, body weight, and testicular weight) as independent variables to analyze their relationship with seminal characteristics, including volume, mass motility, concentration, total sperm number, pH, percentage of live sperm, and percentage of abnormal sperm. Stepwise regression is employed to eliminate factors with minimal impact on the dependent variables

Results

Effect of collection methods on semen characteristics: The effect of collection methods on semen characteristics is presented in Table 1, 2 and 3. The results indicated that both methods are suitable for semen collection in rams. However, the artificial vagina method produced superior semen quality in

terms of concentration, total sperm count, mass motility, and percentage of live sperm compared to electro-ejaculation (Table 1). Significant differences were observed in semen volume and pH between the collection methods ($p < 0.05$; $p < 0.001$, respectively). Semen volume collected via electro-ejaculation (1.19 ± 0.05 ml) was significantly higher than that collected using the artificial vagina method (0.91 ± 0.03 ml) ($p < 0.05$). Furthermore, semen collected via electro-ejaculation exhibited an alkaline pH (7.52 ± 0.07) ($p < 0.001$), whereas that collected via the artificial vagina showed a slightly acidic pH (6.75 ± 0.03). However, microscopic evaluation of sperm morphology was not influenced by the collection methods ($p > 0.05$). While the semen collected via the artificial vagina (AV) method generally shows higher values for major abnormalities compared to electro-ejaculation (EE), particularly significant for the percentage of proximal cytoplasmic droplets and percentage of crater defects.

Effect of season on semen characteristics: The effect of season on seminal characteristics is presented in Table 1, 2, and 3. In fresh semen samples, the season of collection significantly influenced sperm quality. The percentage of abnormal sperm was higher ($p \leq 0.05$) during the non-breeding season, suggesting potential seasonal effects on sperm morphology. Conversely, the percentage of live sperm was significantly greater ($p \leq 0.001$) when ejaculates were obtained during the breeding season, indicating better sperm viability during this period. However, other seminal parameters such as volume, mass motility, concentration, total sperm count, and pH did not show significant differences between the breeding and non-breeding seasons ($p > 0.05$). While there are notable differences in major abnormalities between the breeding and non-breeding seasons (Table 2 and 3). Non-breeding season samples tend to exhibit higher PSAM values across most categories compared to the breeding season.

Effect of age on semen characteristics: The changes in semen characteristics by age in Ouled Djellal rams are shown in Tables 1, 2, and 3. Our results indicate that seminal characteristics did not exhibit significant differences between bulls aged 2 years and 4 years ($p > 0.05$ for all parameters). However, there is minimal variation observed in major abnormalities between rams aged 2 years and those aged 4 years.

Effect of testosterone levels on semen characteristics: The effect of season and testosterone levels on semen characteristics of Ouled Djellal rams is presented in Tables 1, 2, and 3. The present study shows that there were no significant differences in seminal characteristics between bulls with testosterone levels ≤5 ng/ml and >5 ng/ml.

Effect of body weight and testicular weight on semen characteristics: The effects of body weight and testicular weight on semen characteristics of Ouled Djellal rams are detailed in Tables 1, 2, and 3. The current study demonstrates that both body weight (>90 kg vs. ≤90 kg) and testicular weight (>800 g vs. ≤800 g) did not significantly influence seminal characteristics.

Stepwise analyses: Table 4 presents regression parameters, including the coefficient of determination (R2) and the standard error of the estimate (SEE) for predicted traits (Y) associated with sperm characteristics. The results presented in Table 4 show that the method of collection was the most significant predictor for determining sperm volume (ml), with an R2 of 62.50% and a low standard error of the estimate (SEE) of 0.502. This indicates that 62.50% of the variation in sperm volume can be attributed to the method of collection. All other variables were excluded from the prediction model for sperm volume.

The results from Table 4 highlight that the method of collection emerged as the most significant predictor positively and strongly correlated with mass motility ($r = 0.697$, $p \leq 0.0001$), explaining 48.60% of the variation with a low standard error of the estimate (SEE) of 0.223.

Furthermore, the method of collection was found to be the primary predictor for sperm concentration and total sperm count, both showing positive correlations, with R2 values of 62.7% and 44.20%, and SEEs of 1.105 and 1.569, respectively. Other variables were excluded from the model due to their negligible contributions.

Model 2, identified as the best model ($p \leq 0.003$), indicated that the method of collection and season were significant predictors for pH and percentage of live sperm (PSL), with R2 values of 41.90% and 37%, respectively, and standard errors of the estimate (SEEs) of 0.374 and 10.959. Initially, 'method of collection' entered the model first, and 'season' was subsequently added. Variables such as 'age,' 'testosterone level,' 'body weight,' and 'testicular weight' were found to be insignificant and were removed from the model.

In our study, season emerged as a significant factor associated with the percentage of abnormal sperm ($r = 0.459$; $p = 0.018$), indicating that seasonal variations impact sperm morphology. Other variables were excluded from the model, resulting in the equation obtained from the stepwise regression model: PSA = 0.532 + 1.058 × season. This finding underscores the importance of considering seasonal effects when assessing sperm quality in Ouled Djellal ram.

Table 1 Effects of collection methods, season, age, testosterone levels, body weight, and testicular weight on semen characteristics (presented as X ± SEM).

Factors	Seminal characteristics						
	V	MM	C	NTS	pH	PSL	PSA
Collection method							
AV	0.91±0.03*	2.91±0.16***	3.21±0.18***	3.17±0.25***	6.75±0.03***	54.63±2.43***	22.05±2.1ns
EE	1.19±0.05*	1.24±0.14***	0.36±0.02***	0.40±0.04***	7.52±0.07***	43.99±2.61***	22.16±1.8ns
Season							
Breeding	1.02±0.05 ns	2.24±0.19 ns	1.87±0.22 ns	1.93±0.26 ns	6.84±0.06*	54.23±2.64***	20.85±2.0*
Non-breeding	0.94±0.04 ns	2.10±0.15 ns	1.7±0.20 ns	1.63±0.22 ns	7.23±0.08*	44.38±2.43***	23.36±1.8*
Age (years)							
2	0.96±0.04ns	2.09±0.19 ns	1.78±0.22 ns	1.65±0.19 ns	7.16±0.07 ns	47.79±2.40 ns	22.19±1.9ns
4	1.01±0.04ns	2.25±0.16 ns	1.79±0.19 ns	1.92±0.28 ns	7.12±.04 ns	50.83±2.77 ns	22.02±1.1ns
Testosterone							
≤5ng/ml	0.98±0.04 ns	2.18±0.16 ns	1.65±0.18 ns	1.77±0.23 ns	7.14±0.60 ns	48.94±2.36 ns	22.57±1.18 ns
>5ng/ml	1.00±0.05 ns	2.19±0.20 ns	2.04±0.25 ns	1.84±0.26 ns	7.13±0.64 ns	49.67±2.96 ns	20.94±2.12 ns
Body weight							
≤90 kg	0.94±0.05 ns	2.07±0.21 ns	1.58±0.22 ns	1.59±0.25 ns	7.15±0.09 ns	48.56±3.24 ns	23.58±2.45 ns
>90 kg	1.00±0.04 ns	2.23±0.15 ns	1.91±0.19 ns	1.90±0.23 ns	7.13±0.06 ns	49.76±2.19 ns	21.22±1.68 ns
Testicular weight							
≤800 g	0.91±0.04 ns	2.01±0.19 ns	1.50±0.21 ns	1.46±0.24 ns	7.16±0.02 ns	48.26±0.07 ns	22.68±2.34 ns
>800 g	1.03±0.03 ns	2.29±0.16 ns	2.00±0.20 ns	2.02±0.23 ns	7.12±0.08 ns	50.08±0.06 ns	21.68±1.70 ns

Notes. V (volume); MM (mass motility); C (concentration); NTS: (total number in sperm); PSL (percentage of live sperm); PSA (percentage of abnormal sperm); NS: not significant, *: significant at $p<0.05$, ** statistically significant at $p<0.01$, *** statistically significant at $p<0.001$.

Table 2 Effects of collection methods, season, age, testosterone levels, body weight, and testicular weight on major and minor abnormalities (X ± SEM).

Factors	Major abnormalities						
	PSAM	PSAM1	PSAM2	PSAM3	PSAM4	PSAM5	PSAM6
Collection method							
AV	7.64±1.21*	2.62±0.92ns	0.50±0.15ns	3.86±0.71ns	0.06±0.04ns	0.58±0.21*	0.00±0.00ns
EE	4.60±0.60*	0.97±0.55ns	0.40±0.12ns	3.18±0.43ns	0.02±0.02ns	0.09±0.05*	0.00±0.00ns
Season							
Breeding	5.10±0.87ns	0.84±0.28**	0.15±0.06**	3.59±0.72ns	0.00±0.00ns	0.49±0.25ns	0.00±0.00ns
Non-breeding	7.14±1.06ns	2.75±0.90**	0.76±0.20**	3.45±0.42ns	0.09±0.04ns	0.18±0.08ns	0.00±0.00ns
Age (years)							
2	6.23±0.78ns	1.45±0.31ns	0.48±0.15ns	3.64±0.57ns	0.06±0.04ns	0.57±0.21**	0.00±0.00ns
4	6.01±1.13ns	2.14±0.91ns	0.43±0.16ns	3.39±0.61ns	0.02±0.02ns	0.09±0.04**	0.00±0.00ns
Testosterone							
≤5ng/ml	6.61±0.95 ns	2.12±0.71 ns	0.51±0.14 ns	3.64±0.57 ns	0.07±0.04 ns	0.33±0.11 ns	0.00±0.00ns
>5ng/ml	5.22±0.92 ns	1.25±0.45 ns	0.35±0.17 ns	3.26±0.58 ns	0.00±0.00 ns	0.35±0.23 ns	0.00±0.00ns
Body weight							
≤90 kg	6.63±1.08 ns	1.45±0.45 ns	0.43±0.18 ns	4.38±0.84 ns	0.13±0.07 ns	0.25±0.12 ns	0.00±0.00 ns
>90 kg	5.82±0.89 ns	2.00±0.47 ns	0.47±0.14 ns	3.00±0.43 ns	0.00±0.00 ns	0.38±0.15 ns	0.00±0.00 ns
Testicular weight							
≤800 g	6.35±1.20 ns	1.14±0.31 ns	0.50±0.18 ns	4.42±0.87 ns	0.04±0.02 ns	0.31±0.13 ns	0.00±0.00 ns
>800 g	5.95±0.93 ns	2.27±0.80 ns	0.42±0.13 ns	2.85±0.33 ns	0.05±0.01 ns	0.35±0.16 ns	0.00±0.00 ns

Notes. PSAM (major abnormalities); PSAM1 (proximal cytoplasmic droplet); PSAM 2 (periforme head); PSM 3 (curly tail or tail wrapped around the head); PSAM 4 (deformation of the intermediate piece); PSAM 5 (poor development); PSAM 6 (frequency of crater defects). NS: not significant, ** significant at $p<0.01$.

Table 3 Effects of collection methods, season, age, testosterone levels, body weight, and testicular weight on minor abnormalities (X ± SEM).

Factors	Minor abnormalities						
	PSAm	PSAm1	PSAm2	PSAm3	PSAm4	PSAm5	PSAm6
Collection method							
AV	14.05±1.35^{ns}	1.0460.30^{ns}	6.20±1.09^{ns}	6.15±0.64^{ns}	0.14±0.07^{ns}	0.28±0.05^{ns}	0.21±0.06*
EE	17.40±1.41^{ns}	1.70±0.28^{ns}	8.01±0.88^{ns}	7.77±0.88^{ns}	0.00±0.00^{ns}	0.03±0.03^{ns}	0.00±0.00*
Season							
Breeding	15.46±1.50^{ns}	0.89±0.23*	6.09±1.02^{ns}	7.97±0.92^{ns}	0.05±0.04^{ns}	0.01±0.00^{ns}	0.24±0.02^{ns}
Non-breeding	15.98±1.28^{ns}	1.84±0.34*	8.11±.95^{ns}	5.96±0.58^{ns}	0.10±0.06^{ns}	0.31±0.21^{ns}	0.50±0.06^{ns}
Age (years)							
2	15.38±1.37^{ns}	1.54±0.33^{ns}	7.04±0.85^{ns}	6.98±0.73^{ns}	0.11±0.07^{ns}	0.06±0.03*	0.08±0.02^{ns}
4	16.07±1.42^{ns}	1.20±0.25^{ns}	7.17±0.12^{ns}	7.24±0.83^{ns}	1.02±0.02^{ns}	1.25±0.0*	1.15±0.06^{ns}
Testosterone							
≤5ng/ml	15.68±1.32 ^{ns}	1.13±0.21 ^{ns}	6.82±0.96 ^{ns}	7.34±0.74	0.04±0.05 ^{ns}	0.22±0.16 ^{ns}	0.10±0.03 ^{ns}
>5ng/ml	15.39±1.40 ^{ns}	1.63±0.42 ^{ns}	7.44±0.98 ^{ns}	6.19±0.79	0.03±0.01 ^{ns}	0.05±0.04 ^{ns}	0.11±0.07 ^{ns}
Body weight							
≤90 kg	16.93±1.69 ^{ns}	1.43±0.30 ^{ns}	7.71±0.21 ^{ns}	7.28±0.89*	0.13±0.09 ^{ns}	0.27±0.02 ^{ns}	0.05±0.03 ^{ns}
>90 kg	15.00±1.20 ^{ns}	1.33±0.18 ^{ns}	6.74±0.86 ^{ns}	6.78±0.70*	0.03±0.02 ^{ns}	0.08±0.01 ^{ns}	1.13±0.05 ^{ns}
Testicular weight							
≤800 g	16.31±1.61 ^{ns}	1.33±0.29 ^{ns}	7.19±1.05 ^{ns}	7.44±0.99 ^{ns}	0.08±0.06 ^{ns}	0.26±0.12 ^{ns}	0.04±0.02 ^{ns}
>800 g	15.29±1.24 ^{ns}	1.39±0.29 ^{ns}	7.04±0.95 ^{ns}	6.61±0.62 ^{ns}	0.06±0.04 ^{ns}	0.08±0.02 ^{ns}	0.15±0.05 ^{ns}

Notes. PSAm (minor abnormalities); PSm1 (distal cytoplasmic droplet); PSm2 (detached head); PSm 3 (folded or curled tail to the end); PSAm 4 (small or giant narrow head); PSAm5 (abaxial implantation); PSAm6 abnormal acrosome. ^{NS}: not significant, ** significant at p <0.001.

Table 4 Relative contribution (model R^2), standard error of estimate (SEE) and probability (p) in predicting sperm parameters by the stepwise procedure analysis.

Seminal characteristics	Model	Factor Entered	r	R^2	SEE	P	Regression equation
V(ml)	1	Collection method	0.760	0.625	0.402	p <0.0001	V= 1.653+2.874collection method***
MM	1	Collection method	0.697	0.486	0.223	p <0.0001	MM=4.385-1.474 collection method***
C (x10^9 spz/ml)	1	Collection method	0.792	0.627	1.105	p <0.0001	C= 6.066-2.851collection method***
NTS(x10^9 spz/eja)	1	Collection method	0.665	0.442	1.569	p <0.0001	NTS=5.946-2.772collection method***
pH	1	Collection method	0.628	0.394	0.483	p <0.0001	pH= 5.982+0.774 collection method***
	2	Collection method Season	0.647	0.419	0.374	p <0.0001 p =0.016	pH= 5.692+0.774 collection method*** -0.193 season*
PSL (%)	1	Collection method	0.484	0. 234	21.466	p =0.006	PSL= 65.271-10.639 collection method**
	2	Collection method Season	0.609	0.370	10.959	p =0.003	PSL= 80.042-10.639collection method** -9.847 season**
PSA (%)	1	Season	0.459	0.210	20.959	p =0.018	PSA= 0.532+1.058 season*

Notes. V (volume); MM (mass motility); C (concentration); NTS: (total number in sperm); PSL (percentage of live sperm); PSA (percentage of abnormal sperm); *: significant at p <0.05, ** statistically significant at p <0.01, *** statistically significant at p <0.001.

Discussion

The primary objective of this study was to investigate the influence of several factors—specifically collection method, season, age, testosterone levels, body weight, and testicular weight—on semen quality in Ouled Djellal rams. Until now, these factors have not been studied together to determine which one predominantly affects fresh sperm quality in ovine species. This research stands out as the first to utilize stepwise analysis to determine the relative impact of these factors on sperm quality in Ouled Djellal rams, employing both electroejaculation (EE) and artificial vagina (AV) methods for semen collection. The evaluation of semen quality is critical for accurately selecting superior breeding rams, which is essential for optimizing herd productivity by identifying rams with the highest reproductive potential. The insights gained from this study have the potential to inform the development of new strategies to enhance breeding programs and improve the overall genetic quality of Ouled Djellal rams.

Our findings demonstrate that stepwise regression analysis identified the collection method as a key determinant for sperm volume (62.50%), mass motility (48.60%), sperm concentration (62.70%), and total sperm count (44.20%). However, further work on this aspect is required as no available literature could be traced to compare with the present findings. In addition, to collect semen for artificial insemination, samples obtained using an artificial vagina are preferred, as they yield higher sperm concentrations (resulting in more insemination doses) and a greater percentage of live sperm cells (indicating better semen quality). Ejaculates obtained by EE had higher volume and lower concentration than those collected by AV. These results agree with other authors who compared these collection methods in rams (Marco-Jiménez *et al*., 2005) and bucks (Jiménez-Rabadán *et al*., 2012). EE stimulates accessory sex glands via electrical pulses, which may lead to a decreased concentration of sperm but increased of seminal fluid in the semen sample (Baiee *et al*., 2018). Additionally, urinary losses caused in rams by the retrograde flow of spermatozoa contribute to this effect (Dooley *et al*., 1991). Electroejaculation could affect the ejaculation reflex due to the aggressiveness of these techniques, stress management issues, or insufficient stimulation of the rectal mucosa (Marco-Jiménez *et al*., 2005). In a review of several studies, it was concluded that EE induces physiological, neuroendocrine, and behavioral changes that may indicate a stress response associated with pain (Palmer, 2005). Collecting semen using the AV generally required the active participation of the ram, leading to the involvement of the nervous and endocrine systems. Under the influence of these systems, the gonadotrophic hormones (SSH and ICSH) are generally produced in sufficient quantities to regulate the reproductive system (Malejane al., 2014). There are multiple factors that influence the success of semen collection using an AV. The experience and ability of the technician conducting the sessions are among the most important. The trainer must observe if the male shows interest in the female by sniffing and licking her vulva and head; also, some males spend a longer time courting, and the trainer must understand and allow the male to do so to increase its libido before it mounts. When the male shows no interest in the queen, it should be presented to alternate females before (Ackermann and Lopes, 2020).

Our results illustrate a significant difference between the two semen collection techniques, with the AV resulting in a higher mass motility. The AV method of semen collection yielded semen with a wave motion score of >3, while the EE technique yielded a score of < 3. In general, semen scoring >3 for semen wave motion (on a scale of 0 to 5) is accepted as suitable for artificial insemination or cryopreservation (O'Hara *et al*., 2010). In addition, the lower sperm motility in EE appeared to be due to the toxic effects of urine (Ohl *et al*., 1994).

Our results indicated that semen collected using the EE had a significantly higher pH ($p < 0.05$) compared to the AV technique. The semen pH for the AV technique was 6.75 ± 0.03, which is slightly acidic. The results obtained by Ferdinand *et al*. (2012) in the West African Dwarf goat breed were more alkaline, suggesting that the EE technique leads to acidic buck semen, and this might have a negative impact on fertility. The acidic semen gives an indication of excessive accessory gland secretion due to electrical stimulation in the rectum of the buck (Ramukhithi *et al*., 2011). The excessive accessory glands are responsible for the buffering capacity of semen (Sundararam *et al*., 2007). Latif *et al*. (2005) also reported that in an acidic pH environment, the motility of sperm is affected, probably due to a change in the metabolic activity and a disturbance in the cellular respiration of the sperm. The delay in the processing of fresh undiluted semen has been reported to possibly induce the semen pH to become more acidic due to degradation of fructose by the sperm cells (Zamiri *et al*., 2010).

Our results showed that semen collected using an AV had a higher percentage of live sperm compared to ejaculates collected by electroejaculation, which contrasts with the findings of Malejane *et al*. (2014) in Dorper rams, where no difference was observed. However, Ledesma *et al*. (2014) demonstrated that ejaculates obtained via EE have a higher proportion of seminal plasma, which might contain more antioxidant components. This could explain the better quality of sperm cells in those ejaculates. While sperm cells produce reactive oxygen species (ROS) as part of their normal metabolism, excessive ROS can be detrimental. Overproduction of ROS leads to decreased sperm viability, compromised acrosomal integrity, DNA disruption, and mitochondrial deterioration (Aitken and Koppers 2011).

Nevertheless, our findings indicated that no differences in the percentage of abnormal sperm were observed between collection methods, consistent with findings by Marco-Jiménez *et al*. (2005) in Guirra rams, Bopape *et al*. (2015) in South African Indigenous goats, which suggested that the method of sperm collection does not influence sperm morphology.

Our results showed no significant differences in ejaculate characteristics, including ejaculate volume, sperm concentration, and total sperm number, regardless of the collection season. These findings align

with those of Ntemka *et al*. (2019) for Chios ram, Pérez and Mateos (1996) for Malagueña bucks, and Jiménez-Rabadán *et al*. (2012) for Blanca-Celtibérica bucks. Testicular weight remained constant during our experimental period and was not affected by seasonal variations. Therefore, the consistent semen volume obtained across different seasons indicates a positive correlation between semen volume and testicular weight. Although spermatogenesis is active throughout the year in rams, considerable seasonal variations have been reported in different regions (Kridli *et al*., 2007).

Our results indicated that only ejaculates collected during the breeding season exhibited a higher percentage of live sperm, a lower percentage of abnormal sperm, and a lower pH. This was supported by stepwise regression analysis, which identified the season as the second predictor, following the method of collection, in determining the variation of seminal characteristics. These results agree with findings by Al-Anazi *et al*. (2017) in Naimi and Najdi rams and Ngcobo *et al*. (2020) in Zulu rams. Zamiri *et al*. (2010) demonstrated that the effect of photoperiod on seasonal breeders largely depends on latitude. At latitudes above 40°N, significant variations in seminal attributes have been recorded, with increased sperm production during periods of decreasing daylight. At latitudes between 30°N and 40°N, seminal characteristics show less seasonal variation, with higher sperm production observed during the summer and autumn seasons.

Our results indicated that ram age did not significantly influence sperm parameters. This was confirmed by stepwise regression analysis, which did not identify age as a critical predictor of sperm characteristics. These findings suggest that once rams reach sexual maturity, their sperm characteristics remain stable. These results align with Tabbaa *et al*. (2006) who noted that sperm characteristics were not significantly affected by the age of Awassi Rams. These results indicate a well-balanced function of the hypothalamic-pituitary axis, testes, and epididymides, providing efficient support for spermatogenesis in rams across different ages (Ntemka *et al*., 2019).

Few studies have investigated the impact of testosterone on semen analysis to date. Our results showed no significant effects of testosterone levels on sperm production or semen quality. This finding was supported by stepwise regression analysis, which did not identify testosterone levels as a critical predictor of sperm characteristics. This is consistent with the findings of Guardo *et al*. (2020), where multivariable regression analysis revealed that testosterone did not have a significant effect on semen volume, sperm count, motility, or morphology in the men. Albert and Leonardo (2004) showed that an initial rise in Follicle Stimulating Hormone (FSH) leads to the proliferation of Sertoli cells, elongation of the seminiferous tubules, and an increase in tubule diameter. Concurrently, there is a rise in Luteinizing Hormone (LH) secretion, resulting in increased testosterone production by the Leydig cells.

In the present study, the rams were physically mature (2–4 years old) and exhibited minimal variation in body weight and testicular weight during the trial. This was supported by stepwise regression analysis, which did not identify these factors as critical predictors of sperm characteristics. However, no such reports were found available in the literature to compare the present findings. The daily production of spermatozoa is directly correlated with the volume or weight of healthy testicular parenchyma (Abah *et al*., 2023) and the extent of the seminiferous epithelium (Tibary *et al*., 2018). Gouletsou and Fthenakis (2010) demonstrated that if males have a low body condition score after the breeding season, it is advisable to provide a high-energy diet to promote increases in body weight, testicular size, and the number of cells in the germinal layers of the testes, ultimately leading to enhanced sperm production during the non-breeding season. Maquivar *et al*. (2021) demonstrated that preventing excessive fat accumulation is crucial, as rams with a body condition score above 4 may exhibit reduced libido. Additionally, fat accumulation in the scrotum can impair testicular thermoregulation, leading to decreased sperm quality.

In conclusion, after conducting the stepwise regression, this paper makes several contributions. It suggests that the method of collection explains the largest variance in semen production, followed by the season. In terms of predictive accuracy, the artificial vagina outperforms the electro-ejaculator. It is the optimal method for selecting superior breeding rams, ensuring maximum herd productivity, and is suitable for semen production, preservation, and artificial insemination programs. Additionally, there may be a need for a broader experiment involving a larger number of animals to further investigate the influence of other factors such as diet and frequency of semen collection.

Conflicts of interest: The author declares that there is no conflict of interest regarding the publication of this paper.

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