



VARIATIONS IN SEMEN QUALITY PARAMETERS OF OVCHEPOLIAN PRAMENKA RAMS ACCORDING TO THE METHOD OF COLLECTION AND THE METEOROLOGICAL SEASON

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ABSTRACT

The off-breeding season for rams is a time-limiting factor for their use in scientific aims. This research was set upon two aims: (1) to acknowledge the differences of semen quality collected throughout the year, and (2) to investigate which of the two commonly used methods for semen collection (artificial vagina - A.V. and electro ejaculation - E.E.) could prove to be more favorable in the off-breeding period. Five Ovchepolian Pramenka rams were used for this investigation. They were divided in two groups: group 1 (two rams), which was subjected to A.V. method, and group 2 (three rams), which was subjected to E.E. method for semen collection. Semen evaluation included: volume, spermatozoa concentration, live spermatozoa, ejaculate density and motility. According to the season, results have a high statistical significance for the volume ($P<0.01$) and motility ($P<0.001$) parameters. Group 1 and 2 results versification showed a high statistical significance for the motility score ($P<0.001$), ejaculate volume ($P<0.01$) and percentage of live spermatozoa ($P<0.01$) parameters. In conclusion, the A.V. method is more favorable for semen collection in late autumn, winter and spring time when rams are out of the breeding season.

Key words: rams, semen collection, artificial vagina, electro ejaculation, breeding season

INTRODUCTION

Late autumn, winter and spring are considered to be sexual inactive periods for rams in Macedonia (11). Pramenka is considered to be an autochthonous breed for this region. There are three distinct strains of the Pramenka breed: Ovchepolian, Sarplanian and Karakachanska. The Ovchepolian strain accounts for 60% of the total sheep population in Macedonia. Male's average live weight is up to 60 kg, and they are horny type of sheep (1). The mating season is considered to be in autumn when they get to their full sexual potential.

Biological clock and reproductive usability of

these animals in the off season is a limiting factor for their scientific and commercial use. Semen collection in the off season months could be achieved by electro ejaculation (E.E.) (12), but there are some disadvantages that affect the quality of the semen (2, 3, 5, 13, 14). The artificial vagina method (A.V.) is popular and safe way for semen collection in the months when rams are in normal sexual drive (6, 7, 12) and could be also applied to some extent in the off seasonal months (9). There are several scientific articles that make a comparison between these two methods (2, 3, 5, 8), but nonetheless, this field is still wide open to be investigated, including variables such as climate variations and breed of rams.

The first aim of this study was to note the variation in quality parameters of the collected semen, regarding the meteorological seasons of the year, and the method of collection. The second aim was to see whether the E.E. method could prove to

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be as favorable as the A.V method in the off season months of ram sexual activity, regarding the semen quality.

MATERIALS AND METHODS

For the purpose of this research we used 5 rams of the Pramenka breed, which were of the Ovchepolian strain. The rams for our research were around two to three years of age, and their average body weight was 50 kg. They were housed on the premises of the Faculty of Veterinary Medicine – Skopje. The semen collection was undertaken in period of fourteen months, covering four different seasons of the year. The season division was according to the Northern Hemisphere Meteorological Season Division (10).

The rams have been previously trained on artificial vagina collection method. They have been subjected to semen collection twice a week, at least three days apart. Two of the rams have been subjected to artificial vagina method for semen collection (group 1), and the other three have been subjected to electro ejaculation method (group 2). Group 1 has been subjected to the procedure two days in the week with three days apart. Each ram from this group has given 2-3 ejaculates per sampling. Group 2 has been subjected to the E.E. procedure in the same day as group 1. Each ram has given 1-2 ejaculates per sampling in an interval of 15 minutes. The temperature of the water which was used to fill the artificial vaginas was 50-55°C, and the internal vaginal temperature was around 42-43°C. The sterile collection tubes have been warmed at around 35-37°C before their use. A commercial extender for semen (AndroMed®) has been used as lubricant for the inner rubber. The vagina with the attached collection tubes have been immediately packed in an insulator wrap by the time it was transferred to the animal housings, which took around 1 minute.

For the electro ejaculation method, a bipolar electrode was used. The rams were placed in lateral recumbence, and were prepared for the procedure by manual rectal cleansing with saline solution. The prepuce was cleaned with alcohol swab, removing any debris or potential contaminant of the ejaculate. The sigmoid flexure was straightened manually, extending the glans of the penis out of the prepuce, fixating it with a sterile gauze swab. The probe was lubricated, and was gently inserted in the rectum of the ram about 15-20 cm in depth. Directing it

towards the floor of the pelvis, short stimuli were applied in intervals of 4 seconds. The procedure was repeated at least three times until ejaculation was accomplished. The time between each procedure was around 1 minute. Immediately after the ejaculation, the collection tube has been secured in the insulator wrap, protecting the semen of cold shock and sunlight.

After transferring each of the collection tubes in the lab, they were placed in water bath on temperature of 32°C. Each semen sample was evaluated no more than 2 minutes after its collection. The data were written in a special form, which noted the date, used method of semen collection, the number of ram, and all the measured parameters.

Semen volume was assessed from the calibrated collection tubes. Semen density and motility has been evaluated subjectively, scoring from 1 to 5. The scoring system for semen density was as follows: 1: watery ($400-1000 \times 10^6$ sperm/ml); 2: thin milky ($1000-2500 \times 10^6$ sperm/ml); 3: thin creamy ($2500-3500 \times 10^6$ sperm/ml); 4: creamy ($3500-4500 \times 10^6$ sperm/ml); and 5: thick creamy consistency ($4000-6000 \times 10^6$ sperm/ml). Semen motility was assessed by wave motion technique and by manual motility estimation (wet mount). Wave motion is the simplest method for assessing undiluted semen under microscope. For the manual motility estimation, the samples have been diluted in a commercial soy bean based semen extender (AndroMed®) in ratio 1:8. An aliquot of 10 µL was placed on warmed microscope slide and a cover glass was lowered on top of it, avoiding formation of air bubbles. At least 5 widely spaced fields were examined to provide an estimate of the percentage of motile cells. The used scoring system for wave and manual semen motility was as follows: 1: very poor (only 10% of spermatozoa, showed signs of live; only weak movement); 2: poor (no waves were formed but some movement of sperm was visible; 20-40% of spermatozoa were live, but with poor motility); 3: fair (only small, slow moving waves 45-65% of sperm cells were active); 4: good (vigorous wave movement but not as rapid as for score 5; 70-85% of spermatozoa were active) and 5: very good (dense, rapidly moving waves; 90% or more of the spermatozoa were active). Spermatozoa concentration was more precisely assessed using a Neubauer hemocytometer chamber. For this method, 10µL of the fresh semen has been diluted in 1990µL of 3% NaCl solution,

making dilution ratio of 1:200. Twenty μL of the diluted sample was placed under the cover slip of the Neubauer chamber and it was left for 2 minutes until the cells would have stabilized in the fields. The counting was performed in 5 diagonal squares.

Percentage of live spermatozoa has been estimated in fresh semen smears. A drop of fresh semen was placed on a pre-warmed microscope slide (35°C) and then stained with Eosin/Nigrosin (60 μL Eosin/Nigrosin and 6 μL semen) (16). The final percentage of live undamaged spermatozoa, taking in consideration the cell membrane integrity, has been obtained by evaluating at least 100 spermatozoa on different areas of the slide.

The total number of samples ($n=174$) have been divided in two groups, according to the method of collection that was used (group 1: artificial vagina, $n=123$; group 2: electro ejaculation, $n=51$). Each parameter has been compared between these two groups. The statistical significance between the mean values of each group has been evaluated with inferential statistics model, the T test. The equations used for this model was as follows:

$$t \text{ value} = \frac{X_A - X_B}{SE(X_A - X_B)}$$

$$SE(X_A - X_B) = \sqrt{\frac{\text{var } A}{n_A}} + \sqrt{\frac{\text{var } B}{n_B}}$$

* SE=Standard Error of Means; X=mean value; n=number of samples; var=variance

The imbalance in number of samples between the two groups has been excluded as an issue due to the fact that no common conclusions were made

as an overall effect of all parameters. The t test was used only to compare each parameter mean value between the two groups.

RESULTS

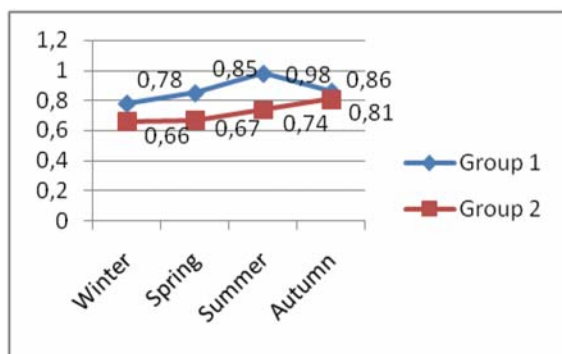
Results according to the season of semen sampling

Table 1 and graphs 1 to 5 show the results of the semen sample analysis, taken in four meteorological seasons with two methods of semen collection. The values for all parameters (volume, concentration of spermatozoa, live spermatozoa, ejaculate density and wave and manual motility) will be respectively presented in the following order of seasons: winter, spring, summer and autumn, versifying between the two methods of semen collection A.V. and E.E. The average values for ejaculate volume (ml) were: 0.78 ± 0.22 vs. 0.66 ± 0.25 , 0.85 ± 0.22 vs. 0.67 ± 0.36 , 0.98 ± 0.22 vs. 0.74 ± 0.24 ($P < 0.01$) and 0.86 ± 0.16 vs. 0.81 ± 0.39 . The values regarding sperm concentration ($\times 10^6$) were as follows: 4117.8 ± 737.77 vs. 3844.4 ± 751.85 ($P < 0.05$), 4868 ± 310.54 vs. 4488.9 ± 631.36 ($P < 0.05$), 4264.5 ± 1032 vs. 4416 ± 818.88 and 4371.4 ± 314.72 vs. 4557.1 ± 544.23 . The percentage of live spermatozoa values were: 85.53 ± 6.6 vs. 81.88 ± 5.77 , 90.24 ± 2.69 vs. 86 ± 8.12 ($P < 0.05$), 90.38 ± 4.82 vs. 85.72 ± 6.92 ($P < 0.01$), and 85.28 ± 9.23 vs. 83.85 ± 10.12 . Ejaculate density parameter showed the following scores: 4.79 ± 0.4 vs. 4.66 ± 0.5 , 5 ± 0 vs. 4.88 ± 0.33 , 4.83 ± 0.45 vs. 4.84 ± 0.37 , 5 ± 0 vs. 4.71 ± 0.48 . Wave and manual motility assessment average scores were: 4.91 ± 0.28 vs. 4.55 ± 0.52 ($P < 0.001$), 5 ± 0 vs. 4.66 ± 0.5 , 4.96 ± 0.17 vs. 4.84 ± 0.37 , and 4.85 ± 0.37 vs. 4.71 ± 0.48 .

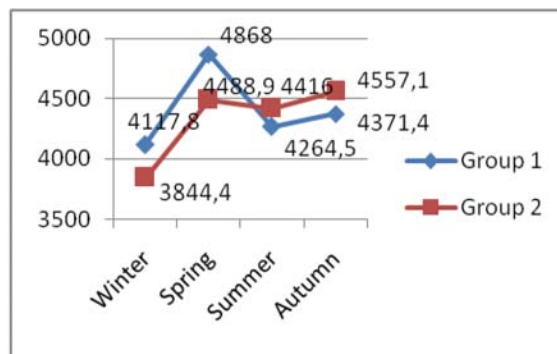
Table 1. Average values for semen quality parameters according to the season of semen coll

SEASON	SEMEN VOLUME (ml.)		SPERMATOZOA CONCENTRATION ($\times 10^6$)		LIVE SPERMATOZOA (%)		SEMEN DENSITY (1-5 score)		SEMEN MOTILITY (1-5 score)	
	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2
Winter	0.78 ± 0.22	0.66 ± 0.25	$4117.8 \pm 737.77^{a\dagger}$	$3844.4 \pm 751.85^{b\dagger}$	85.53 ± 6.6	81.88 ± 5.77	4.79 ± 0.4	4.66 ± 0.5	$4.91 \pm 0.28^{a\dagger}$	$4.55 \pm 0.52^{b\dagger}$
Spring	0.85 ± 0.22	0.67 ± 0.36	$4868 \pm 310.54^{a\dagger}$	$4488.9 \pm 631.36^{b\dagger}$	$90.24 \pm 2.69^{a\dagger}$	$86 \pm 8.12^{b\dagger}$	5 ± 0	4.88 ± 0.33	5 ± 0	4.66 ± 0.5
Summer	$0.98 \pm 0.22^{a*}$	$0.74 \pm 0.24^{b*}$	4264.5 ± 1032	4416 ± 818.88	$90.38 \pm 4.82^{a*}$	$85.72 \pm 6.92^{b*}$	4.83 ± 0.45	4.84 ± 0.37	4.96 ± 0.17	4.84 ± 0.37
Autumn	0.86 ± 0.16	0.81 ± 0.39	4371.4 ± 314.72	4557.1 ± 544.23	85.28 ± 9.23	83.85 ± 10.12	5 ± 0	4.71 ± 0.48	4.85 ± 0.37	4.71 ± 0.48

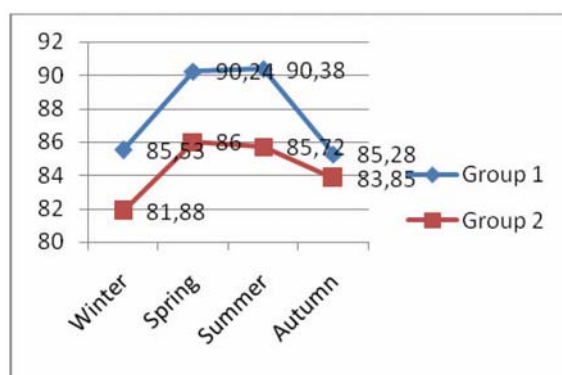
\dagger (a:b) $P < 0.001$; * (a:b) $P < 0.01$; \ddagger (a:b) $P < 0.05$



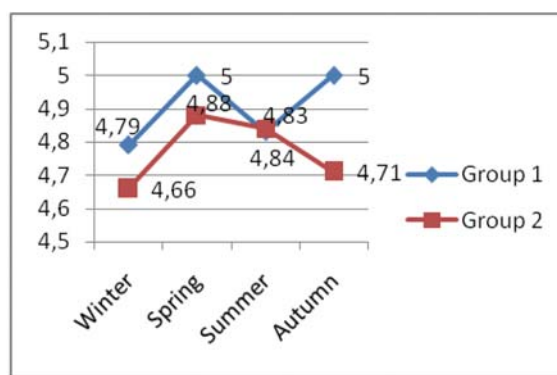
Graph 1. Ejaculate volume (ml) according to the season of semen collection



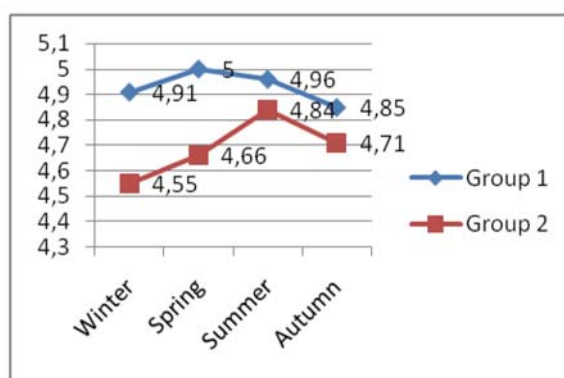
Graph 2. Spermatozoa concentration ($\times 10^6$) according to the season of semen collection



Graph 3. Live spermatozoa (%) according to the season of semen collection



Graph 4. Semen density score (grades 1-5) according to season of semen collection



Graph 5. Semen motility score (grades 1-5) according to the season of semen collection

Results according to the method of collection

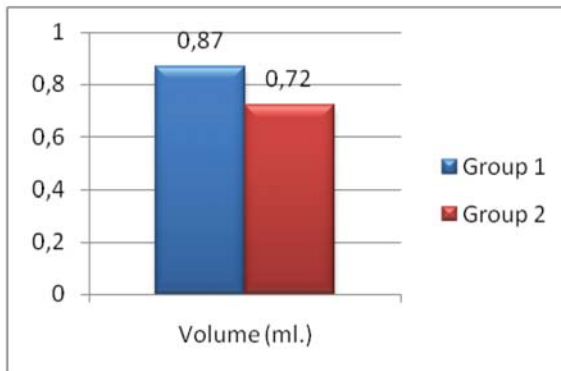
Table 2 and graphs 6 to 10 depict the average summative values from the analysis of the same fertility parameters without consideration of the meteorological seasons. Following the

same parameter order volume, concentration of spermatozoa percentage of live spermatozoa, ejaculate density score and, wave and manual motility score, versifying between the two groups of rams. The results were: 0.87 ± 0.23 vs. 0.72 ± 0.28

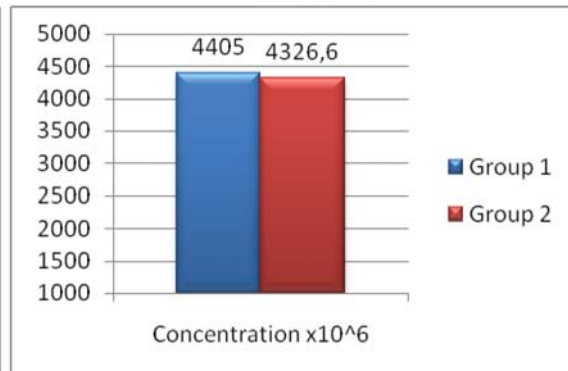
ml ($P<0.01$), 4405.4 ± 795.17 vs. 4326.6 ± 762.17 ($P<0.05$), and 4.93 ± 0.23 vs. 4.69 ± 0.44 (out of 5 scores) ($P<0.001$) respectively.

Table 2. Average values for semen quality parameters according to the method of samplin

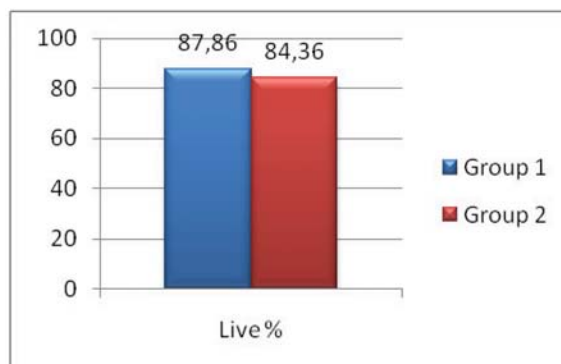
Group	Semen Volume (ml)	Spermatozoa Concentration ($\times 10^6$)	Live Spermatozoa (%)	Semen Density (1-5 grade)	Semen Motility (1-5 grade)
1	0.87 ± 0.23	4405.4 ± 795.17	87.86 ± 6.16	4.9 ± 0.37	4.93 ± 0.23
2	0.72 ± 0.28	4326.6 ± 762.17	84.36 ± 7.39	4.77 ± 0.44	4.69 ± 0.44
P value	$0.004 (<0.01)$	0.5507	$0.0017 (<0.05)$	$0.0423 (<0.05)$	$0.0001 (<0.001)$



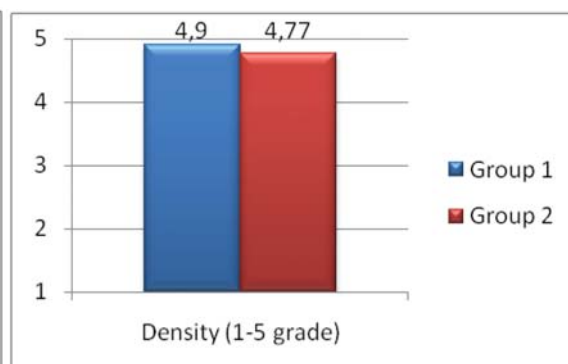
Graph 6. Ejaculate volume (ml) according to method of semen collection



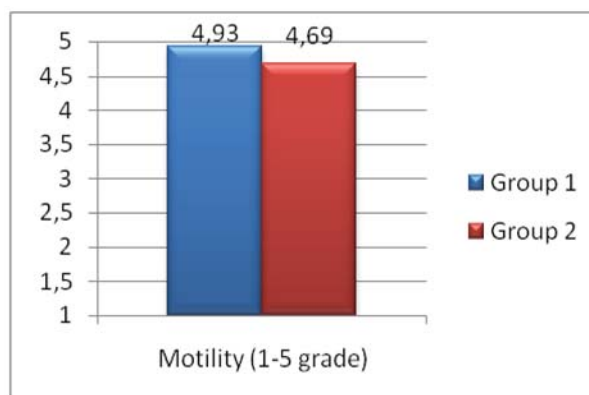
Graph 7. Spermatozoa concentration according to method of semen collection



Graph 8. Live spermatozoa (%) according to method of semen collection



Graph 9. Semen density score (grades 1-5) according to method of semen collection



Graph 10. Semen motility score (grades 1-5) according to method of semen collection

DISCUSSION

The results acquired from the analysis of the samples, taking in consideration the season of the year show a high statistical significance for the volume (summer, $n=56$) and motility (winter, $n=68$) parameters, $P<0.01$ ($P=0.003$) and $P<0.001$ respectively. Results for the percentage of live spermatozoa show moderate statistical significance in the spring period ($n=34$, $P<0.05$). Density score parameter has not shown any statistical significance in any season.

Taking in consideration the two methods of semen collection A.V. and E.E. (group 1 and 2 respectively), there is a very high statistical significance for the motility score ($P<0.001$; $P=0.0001$), and a high statistical significance for the ejaculate volume and percentage of live spermatozoa ($P=0.004$ and 0.0017 respectively), confirming previous reports (3). Density score results have shown a moderate statistical significance ($P<0.05$), while spermatozoa concentration has not shown any, whatsoever, which is in contradiction of a previous investigation (2).

The ejaculate volumes had an obvious variation in its value regarding the two methods of semen collection, and relating to the summer season of the year (2, 3). In the first group, this parameter had increasing tendency starting in the winter and having its peak in the summer, which was for 20.4% higher value than the initial average volume. In autumn this value decreased for 12.24% than the highest value in summer. For the second group, this parameter had growing values throughout the year, reaching its peak in autumn which was for 18.51% higher value than the initial volume in winter.

Spermatozoa concentration in the ejaculates of

the first group reached its pick during the spring, but in the summer it had short decreasing tendency for 12.39%, henceforward continuing with slight rise until the autumn for 6.42%. The second group showed lower values in comparison to the first group, but they had continuous growing pattern, reaching its peak in the autumn, which is for 15.63% higher than the lowest value in winter.

The percentage of live spermatozoa in the ejaculates collected in the first group had growing values from the winter season, up until the summer for 5.21%, thus reaching the pick, and then decreased to a slightly lower value than the samples from the winter season (-0.25%). The second group ejaculates had reached highest values in the spring, rising for 4.79% in comparison to the winter season. In summer and autumn, the values had slightly decreasing tendency for 2.18% from the pick value.

Ejaculate density of the first group had two pick values of the scores in the spring and autumn (5, 5 accordingly). In summer, this value had decreased for 3.2% from the highest value in spring. The initial score in winter was 4.2% lower than the score in spring and autumn. Second group ejaculates had increasing values up until summer for 3.51% from the initial score and then decreased in winter for 2.68%. Interestingly, the ejaculates from the first and second group had almost the same average scores in summer (4.83 ± 0.45 vs. 4.84 ± 0.37 respectively).

Ejaculate wave and manual motility assessment scores for the first group had increasing tendency up until spring, then lowered its value throughout the summer and autumn. The highest score was reached in the spring. The E.E. method ejaculates had growing scores throughout the winter, spring

and summer when it reached its highest grades, and then, in the autumn, it had slight downfall.

Versification of the two groups results show that ejaculate volume, concentration, percentage of live spermatozoa, density and motility scores have by 17.24%, 1.77%, 3.98%, 2.65% and 4.86% higher values in favor of the first group (AV), respectively.

It could be concluded that no matter of the meteorological season, all semen quality parameters show slightly higher values for the A.V. method in comparison to the E.E. method, concluding previous scientific investigations which have reported similar results (2, 3). Other reports have concluded that both methods have advantages and disadvantages on semen fertility parameters, and both could be utilized in semen collection of rams (5). E.E. method of semen collection could be used for acquiring higher volumes of ejaculate (4), but it has some physiological changes in rams that could be contradictory to the Animal Welfare policy in some institutions (6, 15). Nevertheless, A.V. method is proved to be successful in semen collection during the non-breeding period of rams (9), adding to the fact that all fertility parameters are positively influenced.

This report could be concluded by the fact that A.V. is more favorable method for semen collection in rams, even though it could not be utilized in the whole herd due to individual differences in the libido during the non-breeding period. Increasing the number of rams that can be used in the winter and spring periods could be achieved by selecting high-libido individuals of the herd. To justify this hypothesis, further investigations should be made on the influence of whole year utilization on the semen quality during the breeding season.

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