

## EFFECT OF PERCOLL GRADIENT SEPARATION ON SEMEN QUALITY IN RAMS AND ITS POTENTIAL IMPLICATIONS FOR FOOD PRODUCTION

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### Abstract

The quality of semen in small ruminants represents a key indicator of the reproductive potential of breeding males and has an indirect, yet significant, impact on the quality and safety of animal-derived food products. The present study aimed to evaluate the effect of Percoll gradient separation on the main quantitative and kinematic characteristics of spermatozoa in rams (*Ovis aries*) and to analyze the applicability of this technology for targeted management of offspring sex ratio. Semen samples (n=27) were analyzed using a computer-assisted sperm analysis (CASA) system, comparing native ejaculates (n=9) with fractions enriched in X- (n=9) and Y-bearing spermatozoa (n=9) (RX and RY). The data obtained were processed using the open-source JASP software platform (version 0.19.1). The results showed a statistically significant reduction in concentration, total and progressive motility, as well as kinematic parameters (VCL, VAP, VSL) in the sexed fractions compared to the control group (P<0.05). Nevertheless, the RY fraction demonstrated relatively better preservation of progressive motility compared to RX. These findings confirm that Percoll gradient separation affects the functional status of spermatozoa while providing opportunities for the application of reproductive biotechnologies aimed at improving profitability, standardization, and predictability of animal production. The implementation of such approaches in small ruminants may contribute to sustainable sector development and enhance the quality of raw materials within the food chain.

**Key words:** *seminal ejaculates, small ruminants, food quality*

### Introduction

The production of safe and high-quality animal-derived foods is a primary priority in modern animal husbandry and food chain regulatory systems. In small ruminants (sheep and goats), the quality of meat and dairy products is influenced not only by exogenous factors, such

as nutrition and rearing technology, but also by the genetic potential of the population (Han et al. 2025). In this context, the selection of breeding males is established as a fundamental stage in the “farm-to-fork” concept, determining the initial quality parameters of raw materials (Bachev and Terziev, 2017; Teixeira et al. 2020).

Semen quality and the integration of innovative reproductive biotechnologies, such as sperm sexing, provide opportunities for targeted improvement of productive, health, and technological traits in animals, while also preventing sex-linked genetic disorders (Kinga and Piotr, 2026). These technologies are directly correlated with the technological properties of final products, as the genetic architecture of the sire determines key meat production traits: growth rate, muscle hypertrophy, lipid profile, pH stability, and organoleptic characteristics (Hopkins et al. 2011). The use of genetically superior males ensures offspring homogeneity, which is a critical prerequisite for obtaining standardized meat batches with predictable processing qualities (Brito et al. 2017). Selection criteria for breeding males in small ruminants encompass genetic and zootechnical traits, verified origin and pedigree, offspring productivity, selection indices, absence of hereditary and/or reproductive defects, as well as physiological parameters, including proper body development and condition, normal sexual system development, age, and sexual maturity (de Aguiar et al. 2020). Semen quality is a key indicator of the reproductive potential of breeding males and has an indirect yet significant impact on the biological value and safety of products within the food chain (Khan et al. 2024). The main parameters evaluated include ejaculate volume, sperm concentration, total and progressive motility (Figure 1).

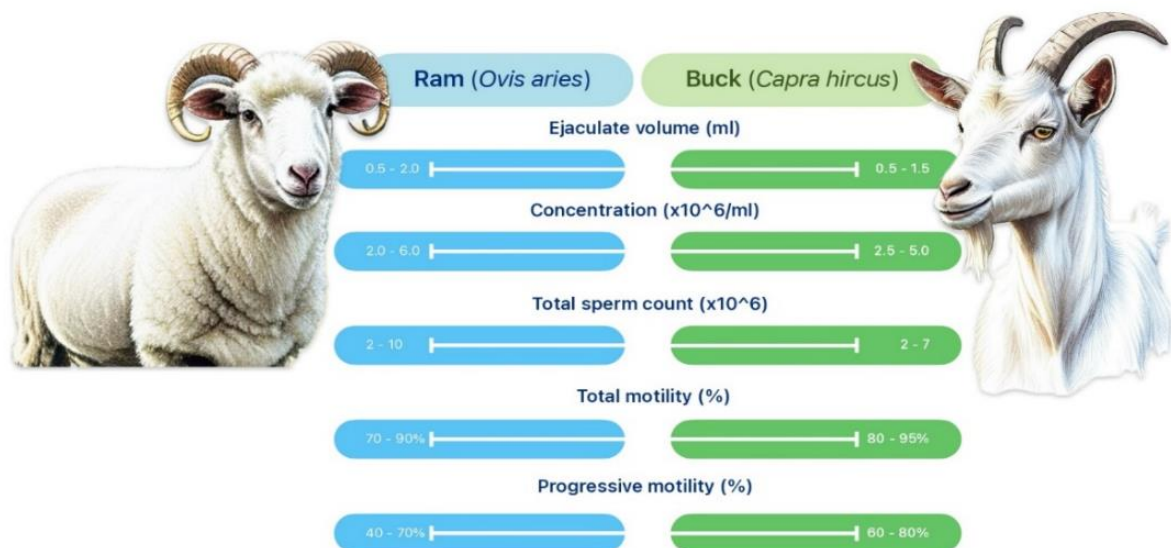


Figure 1. Reference values for key sperm parameters in rams (*Ovis aries*) and bucks (*Capra hircus*) according to established veterinary-andrological standards (Created with Apple Freeform) (adapted from Jha et al., 2018; Herrera Vargas et al., 2023)

In the context of these challenges, the intensification of sheep production requires the implementation of modern reproductive biotechnologies that optimize reproductive efficiency and accelerate genetic progress. Among these, sperm sexing technologies provide opportunities for targeted production of female offspring for flock replacement and for improving productive traits through the selection of genetically superior sires.

### **Sperm Sexing – A Technological Approach Contributing to Food Quality**

Sperm sexing technologies are well established in cattle production, achieving over 90% accuracy in offspring sex prediction while maintaining satisfactory fertility rates (Vishwanath et al. 2018). In contrast, the application of sexed semen in small ruminants remains limited due to technical and economic constraints, including reduced sperm quality after sorting and the need for specialized insemination techniques (De Graaf et al. 2007; Diers et al. 2020). Consequently, the use of sexed semen in sheep breeding is still relatively novel and has not yet been widely implemented in Bulgaria.

Recently, alternative semen sexing methods for small ruminants, such as Percoll density gradient centrifugation (PDGC), have gained increasing attention. This method separates X- and Y-bearing spermatozoa based on density differences, offering a simpler and potentially more accessible approach for practical application (Rahmawati et al. 2025).

Therefore, the present study aimed to evaluate the effect of Percoll density gradient separation on the main quantitative and kinematic characteristics of ram (*Ovis aries*) spermatozoa and to assess its potential as a tool for sex ratio management in sheep production.

## **Materials and Methods**

### *Experimental Design and Bioethical Standards*

The study was conducted using ejaculates (n=9) from clinically healthy breeding rams (*Ovis aries*) reared on farms in the Sofia region, aged 3-5 years maintained under uniform nutritional and management conditions in a commercial farm, following standard animal

welfare practices. All procedures were performed in accordance with European Union Directive 2010/65/EU of the European Parliament and of the Council, concerning the protection of animals used for scientific purposes, as well as in compliance with institutional ethical guidelines. Semen was collected using the standard artificial vagina method and immediately diluted 1:1 with a specialized extender 6A with patent number BG2073U1 to preserve gamete viability during transport and initial processing. It is important to note that the biological material included in the present study was originally collected as part of a larger sample aimed at assessing fertility. Within the framework of the current analysis, however, it is interpreted from a different research perspective – namely, as an indirect marker of food quality.

#### *Assessment of Semen Quality*

Sperm characteristics were analyzed using the Sperm Class Analyzer (SCA, version 6.4.0.99, Microptic, Spain). A 5  $\mu$ L semen sample was placed on a microscope slide and observed under a microscope equipped with a camera connected to specialized software that evaluates sample concentration and quality by analyzing captured images (Amann et al. 2014).

Single-use Leja chamber slides were employed, maintaining a constant temperature of 37°C. Digital images were captured using a phase-contrast microscope at 10 $\times$  magnification, and for each sample, the trajectories of  $\geq 200$  spermatozoa were recorded. The main parameters evaluated included concentration, total and progressive motility, as well as specific velocity parameters: VCL (curvilinear velocity), VSL (straight-line velocity), and VAP (average path velocity). A minimum of 200 spermatozoa per sample was recorded to determine motility and concentration of male gametes (Valverde and Madrigal-Valverde, 2018).

#### *Percoll Gradient Separation of Semen Samples*

For sex-based sperm separation, a modified multilayer Percoll density gradient centrifugation method was applied (Yekti et al. 2024). Samples were divided into three groups: control (untreated), RX (fraction enriched in X-bearing sperm), and RY (fraction enriched in Y-bearing sperm). The process employed a five-layer gradient (20% to 60% density) arranged in descending order in Pyrex tubes. Separation was achieved by centrifugation at 2200 rpm for 5 minutes at 4°C, followed by washing of the isolated fractions. The resulting RX and RY fractions were subsequently re-analyzed using CASA to assess changes in sperm kinetics.

### Statistical Analysis

Data were analyzed using the open-source JASP software platform (version 0.19.1). One-way ANOVA was used to test the null hypothesis and compare mean values among the three groups (control, RX, and RY). Tukey's Honestly Significant Difference (HSD) test was applied to identify specific between-group differences. Results are presented as mean  $\pm$  standard deviation (SD), with a significance level set at  $P < 0.05$ .

### Results

Comparative analysis of sperm parameters revealed a distinct differentiation between the control ejaculates and the fractions subjected to gradient separation (Table 1). Native samples exhibited significantly higher sperm concentration ( $1582.73 \pm 182.39$  M/mL) compared to the isolated RX ( $314.79 \pm 33.67$  M/mL) and RY ( $219.81 \pm 74.12$  M/mL) groups ( $P < 0.009$ ). A similar reduction trend was observed for total motility, which reached  $97.70 \pm 1.24\%$  in the control group, while the sexed fractions decreased to  $50.22 \pm 9.85\%$  for RX and  $47.11 \pm 11.40\%$  for RY ( $P < 0.020$ ).

Progressive motility, a critical factor for fertilizing capacity and semen quality, followed an identical declining pattern: from  $36.09 \pm 4.68\%$  in controls to  $4.71 \pm 0.50\%$  (RX) and  $12.54 \pm 2.47\%$  (RY). Notably, the RY group demonstrated statistically better preservation of progressive motility compared to RX ( $P < 0.038$ ).

The kinematic profile of spermatozoa, assessed via CASA, also showed high sensitivity to the separation process. Curvilinear velocity (VCL) decreased significantly from  $62.63 \pm 4.41$   $\mu\text{m/s}$  in the control group to an average of  $30.5$   $\mu\text{m/s}$  in the treated fractions ( $P < 0.001$ ). Similar significant ( $P < 0.033$ ) deviations were recorded for average path velocity (VAP) and straight-line velocity (VSL). These findings indicate alterations in the energetic status and motility characteristics of gametes resulting from the physicochemical impact during technological processing.

Table 1. Sperm parameter values (mean  $\pm$  SD) in the three studied groups

Parameter	Control group (n=9)	RX group (n=9)	RY group (n=9)
Concentration (M/mL)	$1582.73 \pm 182.39^a$	$314.79 \pm 33.67^b$	$219.81 \pm 74.12^b$
Total motility (%)	$97.70 \pm 1.24^a$	$50.22 \pm 9.85^b$	$47.11 \pm 11.40^b$

<i>Progressive motility (%)</i>	36.09±4.68 <sup>a</sup>	4.71±0.50 <sup>c</sup>	12.54±2.47 <sup>b</sup>
<i>VCL (μm/s)</i>	62.63±4.41 <sup>a</sup>	28.64±2.76 <sup>b</sup>	32.37±6.31 <sup>b</sup>
<i>VAP (μm/s)</i>	32.13±2.47 <sup>a</sup>	13.71±2.25 <sup>b</sup>	15.28±4.14 <sup>b</sup>
<i>VSL (μm/s)</i>	17.31±1.23 <sup>a</sup>	9.55±3.07 <sup>b</sup>	13.87±5.40 <sup>b</sup>

\*Different superscript letters (a, b, c) within the same row indicate statistically significant differences ( $P < 0.05$ ).

\*\*Legend: RX – X-bearing sperm group; RY - Y-bearing sperm group, VCL - Curvilinear velocity; VAP - Average path velocity, VSL - Straight-line velocity.

## Discussion

The present study demonstrates that sperm sexing and separation technologies in sheep induce measurable changes in sperm functional characteristics, while simultaneously representing a promising tool for improving reproductive efficiency and management in small ruminant production systems (Holden and Butler, 2018; De Vries, 2017).

The interpretation of the results was primarily focused on the statistically significant changes observed in sperm kinematic parameters (VCL, VAP, and VSL) following processing. The reduction of these parameters reflects a consistent decline in motility efficiency and altered trajectory dynamics in sex-sorted sperm fractions, indicating compromised functional competence after separation procedures. The observed decreases in VCL, VAP, and VSL can be attributed to the combined mechanical and physicochemical stress associated with centrifugation and density gradient separation. These processes expose spermatozoa to osmotic fluctuations and transient destabilization of membrane structures, which impair sperm motility performance (De Graaf et al. 2007; Diers et al. 2020; Vishwanath and Moreno, 2018; Maxwell and Watson, 1996). At the cellular level, such stressors may disrupt mitochondrial integrity in the sperm midpiece, leading to reduced ATP production, which is essential for sustaining flagellar activity and progressive motility. In addition, alterations in membrane fluidity and ion channel regulation, particularly calcium influx dynamics, can affect flagellar waveform coordination and reduce linear progression efficiency. These mechanisms provide a biological explanation for the observed statistically significant reductions in VCL, VAP, and VSL. Furthermore, that stress is recognized as a key mediator of sperm damage during in vitro handling and centrifugation-based procedures. Increased generation of reactive oxygen species

(ROS) may lead to lipid peroxidation, protein oxidation, and DNA damage, thereby further compromising sperm motility and functional integrity (Al-Khalaifah et al. 2025; Wang et al. 2025). The kinematic parameters are highly sensitive indicators of sperm functional status, with VCL reflecting overall flagellar activity, VAP representing averaged motility efficiency, and VSL indicating directional movement efficiency. The observed reductions in all three parameters suggest a coordinated decline in both energetic level and movement precision following gamete processing (Peris-Frau et al., 2024). Despite these functional impairments, sperm sexing technologies remain of high practical value in sheep production systems. Their application enables targeted control of offspring sex ratio, improved reproductive planning, and enhanced predictability of production outcomes. This contributes to more efficient use of genetic resources and better alignment of production systems with market demands (Holden and Butler, 2018; De Vries, 2017).

Improving processing conditions of spermatozoa in small ruminants could enhance the balance between achieving desired sex selection and maintaining acceptable levels of sperm motility and fertilizing potential.

## Conclusion

The present study demonstrates that although sperm separation technologies may affect the functional and kinematic status of spermatozoa, they represent a strategic tool for the development of the sheep breeding sector. The integration of modern semen evaluation and sexing methods in small ruminants enables targeted sex ratio management, improved production efficiency, and enhanced farm profitability. In addition, these technologies support more consistent productive traits and raw material quality, contributing to the sustainability and competitiveness of the livestock sector.

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