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# EVALUATION OF SEMEN MOTILITY AND MORPHOLOGY IN ROOSTERS OF THE NATIVE KATUNITSA CHICKEN BREED

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## Abstract

This study provides baseline data on semen quality, CASA-assessed motility and sperm morphology in roosters of the endangered Bulgarian Katunitsa chicken breed and evaluates the individual effect. Twenty-four ejaculates from five males were examined; semen was collected by abdominal massage, motility was assessed using a mobile CASA system, and morphology was evaluated on Diff-Quik–stained smears. Mean (±) values were ejaculate volume 0.36 mL; concentration $2.22\times10^9$ spz/mL; total motility 50.8%; progressive motility 19.5%; VCL 113.0 μm/s; VAP 53.7 μm/s; VSL 38.1 μm/s; ALH 6.80 μm; BCF 33.0 Hz; WOB 45.8%. The proportion of morphologically normal sperm was 84.6%, with midpiece and head bends being the most frequent defects. The individual effect was highly significant for all CASA traits and for volume and concentration ($p<0.001$), whereas most morphological traits showed no significant differences among males. Strong positive correlations were observed among average path velocity and straight-line velocity (e.g., VAP–VSL $r=0.981$) and between total and progressive motility ($r=0.872$). Overall, Katunitsa roosters exhibited an active but moderately linear movement pattern and clear individual variation, supporting the integration of CASA profiles into selection decisions and conservation programs for this local genetic resource.

**Keywords:** CASA, Katunitsa chicken, rooster semen, sperm motility, sperm morphology

## INTRODUCTION

In all livestock species, the loss of genetic diversity has been steadily increasing, with poultry genetic resources being among the most threatened. The conservation of biodiversity in domestic animals encompasses the processes of identifying, characterizing, and monitoring genetic resources under the best possible conditions for their short-term utilization, while ensuring their long-term survival. When studying avian biodiversity, it is essential to establish a systematic approach to experimental design and data analysis. Another crucial step for the sustainable management of genetic resources is the implementation of conservation measures

in situ, as living populations, or ex situ, as cryopreserved material (Weigend et al., 2004).

According to FAO (online, 2024), endangered populations should be conserved for their potential future economic use. Threatened populations with economic potential often possess specific adaptations to their native regions or countries of origin, or adaptive abilities that may prove useful in other parts of the world with similar or suitable environmental conditions.

In Bulgaria, animal genetic resources have long attracted attention from both the scientific community and the state authorities, represented by the Executive Agency for Selection and Reproduction in Animal Breeding (IASRG). The first studies in this field were conducted by Acad. Hlebarov (1926). Currently, only two chicken breeds are officially recognized in the country – the Stara Zagora Red and the Black Shumen hen (IASRG, online, 13 January 2024). At the same time, several other local breeds are being studied by researchers but have not yet been officially registered, which makes them particularly vulnerable. These include the Katunitsa chicken, Bregovska dzhinka and Struma chicken (Teneva et al., 2015).

Among these, the Katunitsa chicken has attracted the greatest interest due to its excellent meat quality and relatively high egg production. The breed was created by animal scientist Alexander Nikolov through targeted selection of large local hens from the Plovdiv region based on body size, meat characteristics, and plumage color. By the late 20th and early 21st century, he succeeded in breeding about 400 birds, and in collaboration with Prof. Gerzilov initiated the first studies on productivity and exterior traits of the breed (Nikolov & Gerzilov, 2011). The Katunitsa chicken has been presented at national and international exhibitions, including the European Poultry Exhibition in Metz, France (2015) (Lukanov, 2017).

According to Teneva et al. (2015), Katunitsa chickens are large and well-muscled, with characteristic plumage – grayish copper-red in hens and black-red in roosters. The live body weight of males at 20–21 weeks exceeds 4.40 kg, and that of hens 3.00 kg. Annual egg production reaches 120–150 eggs with an average weight of 60–62 g, making the breed suitable for organic meat production (Gerzilov et al., 2015). At present, three breeding lines are maintained on four farms.

The Katunitsa chicken is one of the few preserved native Bulgarian breeds, well adapted to extensive rearing conditions and characterized by high resilience but small population size and limited selection work. Despite its importance as a genetic resource, data on the semen quality and sperm productivity of roosters of this breed are extremely scarce. There are no published studies describing the CASA-based profile of sperm motility and morphology in Katunitsa chickens, which hampers the application of modern reproductive technologies and conservation programs.

The reproductive capacity of breeding roosters is a key factor determining the efficiency of reproduction in domestic poultry and the maintenance of genetic diversity within populations (Wolc et al., 2019). Semen quality determines not only the fertilizing potential but also the success of artificial insemination, which remains the most practical method for controlled reproduction in chickens. The main parameters used to assess semen productivity include ejaculate volume, sperm concentration, motility, and morphological and kinematic characteristics measured by computer-assisted sperm analysis (CASA) (Li et al., 2025).

CASA systems provide high precision and objectivity in determining sperm velocity, trajectory linearity, and flagellar beat cross frequency (BCF), all of which correlate directly with fertilizing ability (Tesfay et al., 2020). Numerous studies have demonstrated considerable inter-breed variation in CASA parameters among roosters – for example, curvilinear velocity (VCL) ranging from 47 to 82 μm/s and straight-line velocity (VSL) from 20 to 37 μm/s (Di Iorio et al., 2024; Tesfay et al., 2020).

The present study aims to evaluate the main morphological and kinematic parameters of semen in roosters of the native Katunitsa chicken breed using a CASA system for sperm motility analysis. The results will contribute to a deeper understanding of the reproductive characteristics of the breed and provide a basis for future selection and conservation activities.

## MATERIALS AND METHODS

### *Breeding rooster– management and semen collection*

The study was conducted from March to August 2025. A total of 24 ejaculates were collected from five sexually mature roosters of the native Katunitsa chicken breed, reared near the town of Krichim (fig. 1). The region is characterized by a transitional-continental climate with a distinct mountain influence and is located in the foothills of the Rhodope Mountains (300–400 m a.s.l.). The birds were housed individually in pens separated by solid partitions, each with a floor area of 1 m², and had free access to clean drinking water. Feeding was performed with a specially formulated diet for breeding roosters containing metabolizable energy 2866 Kcal/kg and 17% crude protein.

**Figure 1.** Katunitsa chicken breed (orig.).

Ejaculates were collected using the abdominal massage technique according to Burrows and Quinn (1935), in sterile graduated collection cups. Homogenization was performed with a micropipette, and the ejaculate volume was measured using a graduated pipette. Depending on the visual assessment of density, semen was diluted at ratios from 1:45 (for less concentrated) to 1:65 (for more concentrated samples). From each diluted sample, 100 μL were placed in sterile containers for subsequent analyses.

All procedures were carried out at room temperature, including the evaluation of semen using the CASA system; the warming option to 37 °C was not activated. Sperm motility and concentration were analyzed using a mobile CASA system (iSperm mCASA, Aidmics Biotechnology Co., LTD). After careful homogenization, the diluted semen sample was loaded into a disposable chip, which was then inserted into the device for analysis. The software automatically calculated total and progressive motility, sperm concentration, and kinematic parameters.

***Sperm morphology analysis***

Smears for morphological evaluation were prepared immediately after collection to minimize environmental effects on sperm morphology. Ejaculates were diluted 1:5–1:10 with PBS (pH 7.2–7.4) to prevent overlapping of spermatozoa. Two thin smears per sample were made on clean, degreased microscope slides.

Staining was performed using a modified Haema-Schnellfärbung kit (Labor + Technik, Germany), consisting of three reagents: eosin stain (Solution I), fixative (Fix), and basophilic stain (Solution II). To preserve the integrity of the sperm plasma membrane, the fixative solution was omitted. Slides were air-dried at 40 °C for 10 min, stained by immersion for 8 s in Solution I and 12 s in Solution II, rinsed briefly with bidistilled water, drained vertically, and air-dried.

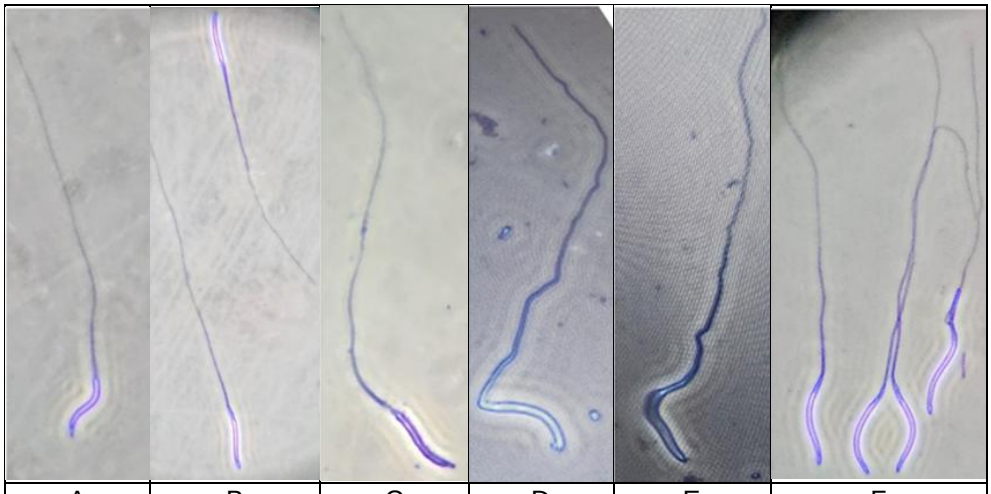


**Figure 2.** Morphological variants of Katunitsa rooster spermatozoa (Haema-Schnellfärbung, ×400) (orig.).
A – Normal spermatozoon; B – Normal spermatozoa; C – Disrupted mitochondrial sheath in the midpiece region; D – Bending of the principal piece at the proximal end; E – Head deformation with zigzag-shaped flagellum; F – Torn mitochondrial sheath in the midpiece (right).

Microscopic observations were performed under a phase-contrast microscope (×400) equipped with a Euromex camera (60 fps) (Figure 2). With this staining method, sperm heads appear dark blue-violet, the acrosomal cap is pale violet to pink, while the midpiece and tail are pinkish-violet to violet. At least 200 spermatozoa

per sample were evaluated and classified into normal or abnormal forms of the head, neck, and tail.

***Statistical analysis***

Data were analyzed using IBM SPSS Statistics 21 and MS Excel 365. The following statistical model was applied:

$Yil = \mu + Ii + eil$

where:

$Yi$ – vector of observations;

$\mu$ – overall mean;

$Ii$– effect of individual (i = 5);

$eil$ – residual variance.

***Ethical approval***

All experimental procedures and animal care followed the recommendations of EU Directive 86/609/EEC and were approved by the Animal Ethics Commission at the Agricultural University – Plovdiv.

## RESULTS AND DISCUSSION

The mean values of the main ejaculate parameters in roosters of the native Katunitsa chicken breed are presented in Table 1. The average ejaculate volume was 0.36 ± 0.03 mL, which falls within the normal range reported for roosters (0.2–0.7 mL; Arif et al., 2025; Zong et al., 2023). The low standard error (SE = 0.031) and moderate standard deviation (SD = 0.174) indicate minimal individual variation. In comparison, the Italian dual-purpose breed Bionda Piemontese shows an ejaculate volume of 0.28 ± 0.05 mL, the highest among 12 native Italian breeds included in the large-scale study of Di Iorio et al. (2024). The same authors reported a mean volume of 0.18 mL, varying widely among breeds—from only 0.03 mL up to 0.61 mL; miniature bantam breeds such as Mericanel della Brianza yield less than 0.1–0.15 mL.

The mean sperm concentration in Katunitsa roosters was 2.22 × $10^9$ spz/mL, occupying an intermediate position compared with some industrial lines such as Barred Plymouth Rock (2.29–3.44 × $10^9$ spz/mL) and Rhode Island Red (1.92 × $10^9$ spz/mL) (Kopec et al., 2025).

The total motility (50.8 ± 5.9 %) was close to the value reported by Tesfay et al. (2020) for White Leghorn roosters (49.66 ± 5.5 %) and markedly lower than that of other breeds (70–96 %) (Di Iorio et al., 2021; Kopec et al., 2025).

The mean progressive motility was fully consistent with the range reported for local Mediterranean breeds (17–33 %; Di Iorio et al., 2024). These results indicate that approximately half of the spermatozoa were motile, but only about one-fifth exhibited a straight-line trajectory.

The kinematic parameters measured by CASA revealed high sperm activity. The curvilinear velocity (VCL) averaged 113.0 µm, which is considerably higher than the typical range for roosters (60–90 µm; Di Iorio et al., 2024), indicating vigorous sperm motion and a high flagellar beat frequency.

The straight-line velocity (VSL), a key indicator of sperm kinetics (Froman & Feltmann, 2000), represents the speed of progression from point A to point B without

regard to the actual trajectory. VSL can therefore be considered the threshold between “mechanical motion” and “functional mobility”, a critical parameter reflecting the fertilizing potential of rooster sperm.

**Table 1.** Main characteristics of semen samples from roosters of the native Katunitsa chicken breed (CASA analysis).

| *Parameter* | *Mean* | ± *SE* | ± *SD* |
|---|---|---|---|
| Ejaculate volume (mL) | 0.363 | 0.031 | 0.174 |
| Sperm concentration (×10⁶ spz/mL) | 2219.9 | 285.6 | 1395.6 |
| Total motility (%) | 50.81 | 5.914 | 27.55 |
| Progressive motility (%) | 19.50 | 4.444 | 20.45 |
| VCL – Curvilinear velocity (μm/s) | 113.0 | 4.884 | 23.29 |
| VAP – Average path velocity (μm/s) | 53.74 | 4.529 | 19.95 |
| VSL – Straight-line velocity (μm/s) | 38.14 | 4.573 | 20.42 |
| LIN – Linearity (%) | 31.53 | 2.674 | 12.07 |
| STR – Straightness (%) | 61.75 | 3.313 | 15.25 |
| ALH – Amplitude of lateral head displacement (μm) | 6.803 | 0.351 | 1.699 |
| BCF – Beat cross frequency (Hz) | 32.97 | 1.089 | 6.374 |
| WOB – Wobble index (%) | 45.75 | 2.332 | 10.30 |

The average path velocity (VAP = 53.74 μm) and VSL (38.14 μm) were 47 % and 66 % lower, respectively, than VCL, illustrating the typical motility pattern in birds active but not fully linear movement. Froman (2006) reported that the minimal VSL required for sperm to pass through the hen’s oviduct must exceed 30 μm. Sayed et al. (2022) observed substantially lower VAP and VSL values in their trials.

Trajectory-shape parameters—linearity (LIN = 31.53 %), straightness (STR = 61.75 %), and wobble index (WOB = 45.75 %)—confirm the predominance of curvilinear trajectories, with only a limited proportion of strictly straight-moving cells.

The amplitude of lateral head displacement (ALH) averaged 6.80 μm, higher than in most breeds (3–5 μm; Madeddu et al., 2024; Li et al., 2025) and indicative of a broad lateral head swing during motion. Combined with the high flagellar beat-cross frequency (BCF = 32.97 Hz), this pattern reflects an active, energetic type of movement typical of spermatozoa with high metabolic activity.

The comparison with literature data shows that in Katunitsa roosters, the sperm velocity parameters (VCL, VAP, VSL) and amplitude of lateral head displacement (ALH) are higher than the average reported for most breeds, whereas linearity (LIN) and progressive motility are within the normal to slightly lower range. The results indicate that roosters of the Katunitsa chicken breed exhibit moderate ejaculate volume and concentration, average total motility, but high sperm velocities and flagellar beat frequency, suggesting good functional activity of spermatozoa.

The morphological characteristics of spermatozoa in Katunitsa roosters are presented in Table 2. The mean proportion of morphologically normal spermatozoa was 84.63 ± 3.95 %, indicating overall good semen quality. This value falls within the upper range of typical values for chickens, as most breeds show 70–90 % morphologically normal spermatozoa (Mavi et al., 2018; Di Iorio et al., 2024). Such

a high level of morphological normality suggests that spermatogenesis and ejaculation proceed physiologically, without pronounced pathological deviations.

Among the defective sperm forms, the most frequent abnormalities were midpiece bending (5.76 ± 2.38 %) and head bending (4.32 ± 0.66 %), which together accounted for over 80 % of all morphological anomalies. Such defects are characteristic of mechanical or thermal damage occurring during sperm maturation or storage in the vas deferens (Froman & Feltmann, 2000).

The midpiece contains the mitochondrial sheath of the spermatozoon and is particularly sensitive to stress factors and temperature fluctuations (Swelum, 2022; Janošíková, 2023).

The observed proportion of morphologically abnormal spermatozoa was below the maximum acceptable threshold for the species (≤ 20 %) (Arif et al., 2025).

**Table 2.** Morphology of spermatozoa in roosters of the native Katunitsa chicken breed.

| *Morphological parameter* | *Mean* | *± SE* | *± SD* |
|---|---|---|---|
| Normal spermatozoa (%) | 84.630 | 3.953 | 16.05 |
| Torn acrosome (%) | 0.372 | 0.196 | 0.780 |
| Bent acrosome (%) | 0.982 | 0.264 | 1.021 |
| Head bending (%) | 4.315 | 0.662 | 3.145 |
| Midpiece bending (%) | 5.759 | 2.377 | 9.251 |
| Midpiece detachment (%) | 0.565 | 0.641 | 2.372 |
| Main-piece rupture (%) | 0.357 | 0.334 | 1.225 |
| Tail detachment at the main-piece region (%) | 0.313 | 0.299 | 1.091 |
| Main-piece bending (%) | 3.125 | 1.721 | 6.470 |
| Main-piece rupture (%) | 0.030 | 0.062 | 0.216 |
| Tail detachment at the distal end (%) | 0.357 | 0.277 | 1.052 |
| Complete tail detachment (%) | 0.060 | 0.079 | 0.296 |
| Agglutination (%) | 2.024 | 0.413 | 2.275 |
| Coagulation (%) | 0.804 | 0.434 | 1.949 |

Other defects, such as acrosomal bending (0.98 %) and midpiece detachment (0.57 %), occurred rarely. The same applies to tail detachment, both at the main-piece and distal regions (0.36 %), which are typically associated with mechanical damage during ejaculation or smear preparation. The low percentage of severe structural abnormalities is a positive indicator of normal spermatogenesis and of the resilience of spermatozoa to external influences.

Agglutination (2.02 %) and coagulation (0.80 %) were also weakly expressed.

Kopec et al. (2025) reported average proportions of morphologically normal spermatozoa between 84 % and 91 % in Plymouth Rock, Rhode Island Red, and Sussex lines, whereas in some local breeds the values are often below 80 % (Emeka et al., 2022).

Our results confirm that in Katunitsa roosters, the sperm morphology is stable and shows limited individual variation, which indicates a good reproductive potential. In comparison with literature data, the morphological profile of Katunitsa roosters can be defined as very good.

Overall, the morphological pattern of semen in Katunitsa roosters is characterized by a high proportion of normal spermatozoa and a low incidence of structural defects.

In combination with the high kinematic values (Table 1), these findings confirm the good reproductive capacity of the roosters and the potential of the breed for efficient use in breeding programs.

The influence of individual males on ejaculate parameters in Katunitsa roosters was highly significant for almost all kinematic traits (Table 3). No significant differences ($p > 0.05$) were observed among roosters regarding morphological parameters, indicating a relatively homogeneous sperm morphology within the population. Exceptions were found for nuclear bending ($p = 0.048$) and agglutination ($p = 0.009$), suggesting individual-specific differences.

Similar individual variations in sperm morphology have also been reported for other native chicken breeds (Emeka et al., 2022). All remaining ejaculate traits volume, concentration, motility, and CASA-derived kinematic parameters, showed a highly significant individual effect ($p < 0.001$).

The highest F-values were recorded for BCF ($F = 917.26$), VCL ($F = 535.41$), WOB ($F = 384.68$), and ALH ($F = 375.66$), confirming substantial individual differences in the velocity and trajectory pattern of sperm movement. Significant individual effects were also observed for ejaculate volume ($F = 133.76$; $p < 0.001$) and sperm concentration ($F = 141.31$; $p < 0.001$), indicating that the intensity of spermatogenesis also varies among males.

## CONCLUSIONS

Roosters of the native Bulgarian Katunitsa chicken breed are characterized by good overall semen quality, expressed through a high proportion of morphologically normal spermatozoa (84.6%) and a low frequency of structural defects. The most common abnormalities were midpiece bending (5.76%) and head bending (4.32%), which did not significantly affect the functional activity of the spermatozoa.

The mean values of ejaculate volume (0.36 mL), sperm concentration ($2.22 \times 10^9$/mL), total motility (50.8%), and progressive motility (19.5%) indicate that the breed maintains a physiologically normal reproductive profile, comparable to other native and industrial chicken lines.

The CASA analysis revealed high sperm velocities (VCL = 113.0 µm/s; VSL = 38.1 µm/s; VAP = 53.7 µm/s) and a high flagellar beat-cross frequency (BCF = 32.97 Hz), reflecting active and metabolically intensive sperm motility.

A highly significant individual effect ($p < 0.001$) was observed for all kinematic parameters, as well as for ejaculate volume, concentration, and motility. This confirms the presence of distinct individual differences among roosters, which should be considered when performing breeding evaluation of sires.

**Table 3.** Effect of individual males on semen parameters in roosters of the native Katunitsa chicken breed.

| *Parameter* | *F-value* | *Significance (p)* |
|---|---|---|
| Normal spermatozoa (%) | 1.616 | 0.234 |
| Torn acrosome (%) | 1.449 | 0.278 |
| Bent acrosome (%) | 1.188 | 0.365 |
| Head bending (%) | 3.312 | 0.048 |
| Midpiece bending (%) | 1.241 | 0.345 |
| Midpiece detachment (%) | 0.836 | 0.528 |
| Head rupture (%) | 0.765 | 0.568 |
| Detached head (%) | 0.722 | 0.593 |
| Head fracture (%) | 0.957 | 0.465 |
| Distal bending (%) | 0.388 | 0.813 |
| Distal detachment (%) | 1.043 | 0.425 |
| Decapitated sperm (%) | 0.971 | 0.459 |
| Agglutination (%) | 5.499 | 0.009 |
| Coagulation (%) | 2.647 | 0.086 |
| Ejaculate volume (mL) | 133.763 | <0.001 |
| Concentration (×10⁹ spz/mL) | 141.310 | <0.001 |
| Total motility (%) | 73.809 | <0.001 |
| Progressive motility (%) | 19.251 | <0.001 |
| VCL (µm/s) | 535.413 | <0.001 |
| VAP (µm/s) | 140.800 | <0.001 |
| VSL (µm/s) | 69.589 | <0.001 |
| LIN (%) | 139.084 | <0.001 |
| STR (%) | 347.425 | <0.001 |
| ALH (µm) | 375.662 | <0.001 |
| BCF (Hz) | 917.262 | <0.001 |
| WOB (%) | 384.678 | <0.001 |
| Raw concentration (×10⁹ spz/mL) | 60.427 | <0.001 |


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