

RESEARCH ARTICLE

Effects of repeated ejaculation on sperm kinematics, seminal plasma biochemical composition, and protein molecular-weight profiles in tropical Saanen bucks



Annisa Rahmi^{1,2} , Ni Wayan Kurniani Karja³ , Mokhamad Fakhrol Ulum³ , Syahrudin Said⁴ , Rahadian Pratama⁵ ,
Abdullah Baharun² and Raden Iis Arifiantini³

1. Doctoral Program of Animal Biomedical Science, School of Veterinary Medicine and Biomedical Sciences, IPB University, Bogor 16680, West Java, Indonesia.
2. Department of Animal Husbandry, Faculty of Agriculture, Universitas Djuanda, Jl. Tol Ciawi No. 1, Bogor, West Java 16720, Indonesia.
3. Division of Reproduction and Obstetrics, School of Veterinary Medicine and Biomedical Sciences, IPB University, Bogor 16680, West Java, Indonesia.
4. Research Center for Applied Zoology, National Research and Innovation Agency (BRIN), Jl. Raya Bogor KM 46, Cibinong, West Java 16911, Indonesia.
5. Department of Biochemistry, Faculty of Mathematics and Natural Science, IPB University, Bogor 16680, West Java, Indonesia.

ABSTRACT

Background and Aim: Ejaculation frequency is an important consideration in natural mating and semen collection programs because repeated ejaculation may alter seminal plasma composition and sperm function. However, integrated information on these responses in Saanen bucks maintained under tropical conditions remains limited. This study evaluated how repeated ejaculation affects fresh semen quality, sperm kinematics, seminal plasma biochemical components, and protein molecular-weight profiles in tropical Saanen bucks.

Materials and Methods: Six Saanen bucks aged 2–3 years underwent a 7-day sexual rest period before semen collection. Five successive ejaculates (E1–E5) were collected from each buck within a single day, yielding 30 ejaculates. Fresh semen quality was assessed using standard laboratory procedures, and sperm motility and kinematics were evaluated using computer-assisted sperm analysis. Seminal plasma biochemical components were quantified spectrophotometrically, whereas protein molecular-weight fractions were characterized using one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Associations between ejaculation frequency and semen or biochemical variables were evaluated using Pearson's correlation analysis.

Results: Ejaculation frequency did not affect most conventional semen quality parameters, including total and progressive motility, sperm viability, plasma membrane integrity, and acrosome integrity ($p > 0.05$), whereas semen pH differed significantly ($p < 0.05$). Among sperm kinematic variables, distance, average path, and amplitude of lateral head displacement differed significantly, while velocity-related parameters remained relatively stable. Five major seminal plasma protein fractions of approximately 15, 20, 30, 35, and 50 kDa were identified, with the approximately 15-kDa fraction showing the greatest relative abundance. Ejaculation frequency was negatively correlated with semen color ($r = -0.488$, $p = 0.005$), total protein ($r = -0.945$, $p = 0.015$), albumin ($r = -0.964$, $p = 0.008$), cholesterol ($r = -0.937$, $p = 0.019$), and triglycerides ($r = -0.969$, $p = 0.006$).

Conclusion: Repeated ejaculation up to five times within a day largely preserved conventional fresh semen quality in tropical Saanen bucks but was associated with changes in sperm movement characteristics and marked reductions in key seminal plasma biochemical components. These findings indicate that seminal plasma composition may be more sensitive than conventional semen traits to increased ejaculation frequency and should be considered when optimizing breeding and semen collection schedules.

Keywords: ejaculation frequency, seminal plasma biochemistry, seminal plasma proteins, sperm kinematics, sperm motility, sperm quality, Saanen bucks, tropical environment.

Corresponding Author: Raden Iis Arifiantini

E-mail: arifiantini@apps.ipb.ac.id

Received: 11-06-2026, **Accepted:** 28-09-2026, **Published online:** 29-10-2026

Co-authors: AR: annisa.rahmi@unida.ac.id; NWKK: karja_nwk@apps.ipb.ac.id; MFU: ulum@apps.ipb.ac.id; SS: syahrudinsaid01@gmail.com; RP: rahadian@apps.ipb.ac.id; AB: abdullah.baharun@unida.ac.id

How to cite: Rahmi A, Karja NWK, Ulum MF, Said S, Pratama R, Baharun A, *et al.* Effects of repeated ejaculation on sperm kinematics, seminal plasma biochemical composition, and protein molecular-weight profiles in tropical Saanen bucks. *Vet World*, 2026, 19(10): 4857–4873.

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INTRODUCTION

Dairy goats have received increasing attention as a practical alternative to dairy cattle in tropical regions because of their adaptability [1], efficient feed utilization [2], and relatively rapid reproductive cycles [3]. Among dairy goat breeds, Saanen goats are widely recognized for their high milk production [4] and have increasingly been introduced into tropical production systems [5]. However, Saanen goats originated in temperate regions, and their reproductive responses may differ under tropical conditions characterized by high ambient temperatures and humidity. These environmental conditions may impose additional physiological challenges on male reproductive function, making the evaluation of semen characteristics in tropical Saanen bucks particularly relevant to breeding management. Male reproductive efficiency is a key determinant of herd productivity in dairy goat systems [6], especially when breeding relies primarily on natural mating [7]. The use of artificial insemination remains limited in goats and is generally associated with lower fertility than in cattle [8] because of the anatomical complexity of the caprine cervix [9], sperm sensitivity to handling and cryopreservation [10, 11], and difficulties in estrus detection [12]. Buck semen is susceptible to oxidative stress during processing because of its low ejaculate volume and high sperm concentration, which may limit seminal plasma-derived antioxidant protection and compromise sperm membrane integrity and fertility [13]. Temperature fluctuations during semen handling, cooling, and cryopreservation may further disrupt sperm membrane structure and function, and conventional semen quality parameters may not readily detect these alterations [14]. Consequently, natural mating remains an important breeding method in dairy goat production systems, including those involving Saanen bucks raised under tropical conditions.

Under natural mating systems, a buck-to-doe ratio of approximately 1:10–1:20 may expose an individual buck to 10–20 potential mating opportunities during a mating period, representing a substantial reproductive workload [15]. Repeated ejaculation may affect semen characteristics through changes in sperm reserves and seminal plasma composition. Previous studies in rams have demonstrated relatively stable semen quality following repeated ejaculation [16]. However, studies in goats have predominantly focused on conventional semen characteristics and have not comprehensively evaluated concurrent changes in seminal plasma composition and sperm kinematics. Furthermore, findings from goats maintained under temperate or non-tropical conditions may not directly apply to Saanen bucks raised in tropical environments.

Seminal plasma provides a complex biochemical environment that supports sperm motility, metabolism, membrane stability, and fertilizing capacity [17]. Seminal plasma proteins also influence sperm kinematics and mediate sperm interactions within the female reproductive tract [18]. Consequently, changes in the molecular-weight distribution and relative abundance of seminal plasma proteins may accompany functional changes in spermatozoa. Advances in one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1D-SDS-PAGE) and computer-assisted sperm analysis (CASA) have facilitated the characterization of seminal plasma protein profiles and objective assessment of sperm movement characteristics, respectively [19, 20].

Despite the recognized importance of ejaculation frequency in breeding management, the physiological responses of tropical Saanen bucks to repeated ejaculation remain insufficiently characterized. Existing studies have largely emphasized conventional semen quality parameters, whereas information on concurrent responses in sperm kinematics, seminal plasma biochemical constituents, and seminal plasma protein molecular-weight fractions remains limited. In particular, it remains unclear whether repeated ejaculation alters the biochemical environment supporting sperm function even when conventional indicators of semen quality remain relatively stable. The effects of ejaculation frequency on the molecular-weight distribution and relative abundance of seminal plasma protein fractions in tropical Saanen bucks are also poorly understood. Moreover, the limited integration of conventional semen evaluation, CASA-derived sperm kinematics, seminal plasma biochemistry, and protein profiling restricts a comprehensive understanding of how repeated ejaculation influences male reproductive physiology under tropical production conditions. Addressing these knowledge gaps is essential to establishing evidence-based mating and semen collection strategies for Saanen bucks under intensive reproductive use.

Therefore, this study aimed to evaluate the effects of repeated ejaculation on fresh semen quality, sperm kinematics, seminal plasma biochemical composition, and seminal plasma protein molecular-weight profiles in Saanen bucks maintained under tropical conditions. Five successive ejaculations collected within a single day were used as a controlled repeated ejaculation model to characterize progressive responses from the first to the fifth ejaculation. The study further examined associations between ejaculation frequency and semen quality and

seminal plasma biochemical variables to determine whether changes in the seminal plasma environment accompany repeated ejaculation. By integrating conventional semen evaluation with CASA-derived sperm kinematics, biochemical measurements, and 1D-SDS-PAGE protein profiling, the study aimed to more comprehensively characterize the reproductive responses of tropical Saanen bucks to repeated semen collection and to generate information relevant to optimizing breeding and semen collection management.

MATERIALS AND METHODS

Ethical approval

All procedures involving animals were conducted in accordance with internationally accepted guidelines for the care and use of animals in research and applicable national and institutional animal welfare regulations. The study protocol, including animal selection, management, handling, semen collection, and associated laboratory procedures, was reviewed and approved by the Animal Ethics Committee of the School of Veterinary Medicine and Biomedical Sciences, IPB University, Indonesia (Approval Certificate No. 400/KEH/SKE/XI/2025). Throughout the study, appropriate measures were implemented to minimize animal discomfort and stress. The bucks were handled by experienced personnel, and semen was collected using an artificial vagina under controlled conditions. No invasive procedures were performed, and the animals were monitored throughout the experimental period to ensure their health and welfare.

Study period and location

Semen collection was conducted at the BBPTUHPT Baturraden facility in January 2026. Subsequent seminal plasma protein profiling and biochemical analyses were performed in February 2026 at the Genomic Laboratory of the National Research and Innovation Agency (BRIN), Cibinong, West Java, Indonesia. The study was conducted at the Balai Besar Pembibitan Ternak Unggul dan Hijauan Pakan Ternak (BBPTUHPT) Baturraden, Central Java, Indonesia, located approximately 600–725 m above sea level. Ambient temperatures at the study location generally range from 18°C to 30°C, with a relative humidity of 70%–80%.

Study design

This study used a repeated-measures design to evaluate the effects of successive ejaculation on semen characteristics, sperm kinematics, and seminal plasma components. Six Saanen bucks aged 2–3 years, weighing 60–70 kg, and with body condition scores (BCS) of 3.0–3.5 on a 1–5 scale were included. All bucks had previous reproductive experience and had successfully produced offspring through natural mating. Before semen collection, the bucks underwent a 7-day sexual rest period to standardize their reproductive status.

Each buck provided five successive ejaculates (E1–E5) within a single day, resulting in 30 ejaculates. The intervals between successive ejaculations varied according to the physiological condition of each buck and ranged from 5 to 373 min was acknowledged as a potential source of variability and a limitation of the study. Semen was collected during mid-morning sessions to minimize environmental variation. Immediately after collection, each ejaculate underwent macroscopic and microscopic evaluation, and sperm concentration, sperm motility, and kinematic characteristics were assessed using CASA. After the initial evaluation, samples intended for subsequent analyses were stored at –20°C in insulated containers and transported under controlled conditions to the Genomic Laboratory of BRIN for seminal plasma protein profiling and biochemical analyses.

Semen collection and analysis

Semen was collected using an artificial vagina (AV). Before each collection, an estrous doe was used as a teaser to provide sexual stimulation. Each buck was allowed two false mounts, and semen was collected on the third mount using an AV maintained at 42°C–45°C. Collections were performed during mid-morning hours to minimize variation associated with environmental heat exposure. Five successive ejaculates were obtained from each buck according to the repeated ejaculation protocol described above.

Immediately after collection, semen volume was measured directly using the graduated collection tube, and semen pH was determined using a pH meter (S-610L Laboratory, Peak Instruments Co., Ltd., Shanghai). Semen color was scored as 1 (yellow), 2 (milky), or 3 (creamy). Consistency was scored as 1 (watery), 2 (medium), or 3 (thick). Mass movement was evaluated microscopically from undiluted semen and scored as 1 (+), 2(++), or 3(+++), according to the intensity of the observed wave-like sperm movement. Sperm viability and abnormality were assessed using eosin-nigrosine staining. Viable spermatozoa were identified by their unstained heads, whereas nonviable spermatozoa showed pink to red staining. Sperm abnormalities were evaluated based on

morphological defects. At least 200 spermatozoa per slide were examined under a light microscope to determine sperm viability and abnormality. Plasma membrane integrity was assessed using hypo-osmotic swelling test (HOST) with a hypo-osmotic solution of 150 mOsmol. Spermatozoa exhibiting tail swelling or curling after exposure to the solution were classified as having intact plasma membranes. Acrosome integrity was evaluated using Giemsa staining, and spermatozoa with intact acrosomal caps were classified as having intact acrosomes.

Sperm concentration, motility, and kinematic characteristics were evaluated using a portable CASA system (AndroScope®, Minitube, Germany; Ref. 12500/3000) based on AndroVision® technology and operated using software version 1.1.0.0. During CASA evaluation, samples were maintained at 37°C using the integrated heating stage and loaded into a 20-µm disposable counting chamber (Minitube). A minimum of five randomly selected microscopic fields per sample were captured and analyzed using a 20× phase-contrast objective. The small-ruminant species profile was selected, and the CASA settings were maintained consistently throughout the analyses. The frame rate was 60 Hz, with 30 frames analyzed per field. Spermatozoa were detected using a minimum contrast threshold of 35 and a minimum cell size of 4 pixels. The static cell gate was set at 5.0 µm/s, and spermatozoa with a straightness (STR) value ≥75% were classified as progressively motile.

Fresh semen was diluted with prewarmed phosphate-buffered saline (PBS) to obtain an appropriate concentration for CASA evaluation, targeting approximately 300 spermatozoa per field of view. A 3-µL aliquot was loaded into a prewarmed chamber and analyzed at 37°C. At least five fields were evaluated for each sample. The CASA system was used to determine total motility, progressive motility, curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), STR, amplitude of lateral head displacement (ALH), and beat cross frequency (BCF).

Pooling of semen samples for biochemical and SDS-PAGE analyses

For seminal plasma biochemical and SDS-PAGE analyses, samples were pooled according to ejaculation frequency because the volume obtained from individual ejaculates was insufficient for separate analyses. Semen samples obtained from the six bucks at the same ejaculation frequency were combined; therefore, E1 samples were pooled together, followed by E2, E3, E4, and E5. This procedure generated five pooled samples, each representing one ejaculation frequency.

For biochemical analysis, each parameter was measured in duplicate, and the mean of the two measurements was used as the final value for the corresponding pooled sample. Thus, the biochemical values reported for each ejaculation frequency represented the means of duplicate measurements obtained from the corresponding pooled sample.

Following semen evaluation, seminal plasma was separated by centrifugation at $1,800 \times g$ for 15 min at 4°C to remove spermatozoa and cellular debris. The supernatant was carefully collected and stored at -20°C until biochemical and protein profile analyses.

Protein extraction and 1D-SDS-PAGE fractions

Fresh semen samples were centrifuged at $6,500 \times g$ for 30 min at 4°C to separate spermatozoa from seminal plasma. The seminal plasma fraction was carefully collected, and total protein concentration was measured using the bicinchoninic acid (BCA) assay with the Pierce BCA Protein Assay Kit (Cat. No. 23225, Thermo Scientific, USA). For protein fractionation, samples were separated by 1D-SDS-PAGE using SurePAGE™ Bis-Tris 4-20% precast gels (M00656; GenScript) at 200 V and 100 mA for 45 min. The gels were subsequently stained with Coomassie Brilliant Blue, and molecular weights were estimated using a broad-range multicolor prestained protein standard (5-270 kDa; M00624). The SDS-PAGE analysis was performed in a single experimental run. Gel images were analyzed using GelAnalyzer software (version 23.1.1), and molecular weight estimation was performed by calibration against the protein marker according to the relative migration distance (Rf) of each protein band. Molecular-weight estimates obtained visually from the SDS-PAGE bands were further refined using GelAnalyzer software calibration [21] to quantify the relative volume of each protein band. Relative band volume was calculated as the percentage of the individual band's volume relative to the total volume of the detected bands in the corresponding lane. The 1D-SDS-PAGE analysis was performed once for descriptive purposes, with no independent electrophoretic replicates.

Seminal plasma biochemical components

Samples were centrifuged at $1,800 \times g$ for 15 min at 4°C to separate the seminal plasma. The supernatant was carefully collected and stored at -20°C until analysis. Biochemical components were quantified using

commercial assay kits and spectrophotometric methods. Total protein was measured at 546 nm using a commercial kit (Biomaxima, Poland), albumin at 628 nm (Labkit, Barcelona, Spain), glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) at 505 nm (Labtest, Brazil), total cholesterol at 505 nm and triglycerides at 505 nm (Grainer, Germany), and fructose concentration was determined using a resorcinol-based colorimetric method at 530 nm.

Statistical analysis

Data from individual bucks were analyzed using a repeated-measures approach, with ejaculation frequency (E1–E5) treated as the within-buck factor and buck as the experimental subject. Normality and homogeneity of variance were assessed using the Shapiro–Wilk and Levene’s tests, respectively. Differences among ejaculation frequencies were evaluated using repeated-measures analysis of variance (ANOVA). When a significant overall effect was detected, pairwise comparisons were performed with Holm adjustment for multiple comparisons. A linear mixed-effects model was additionally fitted as a sensitivity analysis, with ejaculation frequency included as a fixed effect and buck as a random intercept.

Pearson’s correlation analysis was performed to assess linear associations between ejaculation frequency and semen quality based on individual observations ($n = 30$), and between ejaculation order and seminal plasma biochemical parameters based on five pooled observations corresponding to E1–E5 ($n = 5$). Correlation coefficients (r) and corresponding two-tailed p -values were calculated, with statistical significance set at $p < 0.05$. All statistical analyses were performed using SPSS Statistics version 33.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Fresh semen quality at different ejaculation frequencies

Fresh semen quality across ejaculation frequencies (E1–E5) is presented in Table 1. Most evaluated parameters did not differ significantly among ejaculation frequencies ($p > 0.05$). Semen volume showed a numerical decrease from 1.39 ± 0.23 mL at E1 to 0.77 ± 0.20 mL at E5; however, this difference was not statistically significant ($p > 0.05$). Semen color and consistency also did not differ significantly among ejaculation frequencies ($p > 0.05$). In contrast, semen pH differed significantly among ejaculation frequencies ($p < 0.05$), with the highest mean value recorded at E2 (6.86 ± 0.11) and the lowest at E5 (6.24 ± 0.14).

Table 1: Fresh semen quality of tropical Saanen bucks at different ejaculation frequencies.

Parameters	E1	E2	E3	E4	E5
Volume (mL)	1.39 ± 0.23^a	1.25 ± 0.41^a	1.08 ± 0.28^a	0.98 ± 0.20^a	0.77 ± 0.20^a
Color	1.71 ± 0.36^a	1.33 ± 0.56^a	1.33 ± 0.42^a	1.33 ± 0.42^a	1.67 ± 0.42^a
pH	6.68 ± 0.08^{ab}	6.86 ± 0.11^b	6.62 ± 0.23^{ab}	6.26 ± 0.18^a	6.24 ± 0.14^a
Consistency	1.86 ± 0.70^a	1.50 ± 0.61^a	1.33 ± 0.54^a	1.33 ± 0.54^a	1.33 ± 0.54^a
Mass movement	2.00 ± 0.76^a	1.33 ± 0.54^a	1.83 ± 0.75^a	1.83 ± 0.75^a	1.67 ± 0.68^a
Sperm concentration ($\times 10^6$ /mL)	2252.11 ± 853.81^a	990.73 ± 428.62^a	1159.21 ± 558.80^a	1479.57 ± 466.61^a	1101.09 ± 337.73^a
TM (%)	60.33 ± 11.32^a	47.14 ± 12.56^a	52.10 ± 12.61^a	66.57 ± 7.98^a	62.52 ± 11.89^a
PM (%)	58.54 ± 11.25^a	45.94 ± 12.59^a	50.67 ± 12.61^a	65.18 ± 8.06^a	59.78 ± 14.49^a
FM (%)	48.58 ± 17.23^a	37.44 ± 15.28^a	40.60 ± 16.57^a	56.09 ± 22.90^a	42.27 ± 17.26^a
SM (%)	3.46 ± 1.31^a	2.79 ± 1.14^a	3.87 ± 1.58^a	2.59 ± 1.06^a	3.59 ± 1.46^a
CM (%)	9.51 ± 3.59^a	5.72 ± 2.33^a	6.62 ± 2.70^a	6.50 ± 2.65^a	4.36 ± 1.78^a
LM (%)	1.87 ± 0.71^a	1.20 ± 0.49^a	1.44 ± 0.59^a	1.39 ± 0.57^a	1.08 ± 0.44^a
Immotile (%)	39.67 ± 14.99^a	52.86 ± 21.58^a	47.90 ± 19.56^a	33.43 ± 13.65^a	48.81 ± 19.92^a
Sperm viability (%)	75.87 ± 5.38^a	71.45 ± 5.60^a	62.85 ± 10.34^a	69.13 ± 11.19^a	73.63 ± 6.15^a
Sperm abnormality (%)	6.25 ± 1.32^a	10.19 ± 2.06^a	12.34 ± 3.74^a	7.99 ± 2.03^a	11.58 ± 6.36^a
MI (%)	50.14 ± 11.04^a	46.06 ± 8.96^a	45.72 ± 11.21^a	49.43 ± 10.49^a	61.62 ± 6.59^a
AI (%)	93.89 ± 1.07^a	91.75 ± 2.47^a	92.85 ± 1.68^a	92.85 ± 1.52^a	88.98 ± 5.07^a

Color: 1 = yellow, 2 = milky, and 3 = creamy; consistency: 1 = watery, 2 = medium, and 3 = thick; mass movement: 1 = +, 2 = ++, and 3 = +++. TM = Total motility; PM = Progressive motility; FM = Fast motility; SM = Slow motility; CM = Circular motility; LM = Local motility; MI = Plasma membrane integrity; AI = Acrosome integrity. Values are presented as mean $\pm$ SE. Different superscript letters within a row indicate significant differences among ejaculation frequencies based on pairwise comparisons with Holm adjustment ($p < 0.05$).

At the microscopic level, ejaculation frequency did not significantly affect mass movement ($p > 0.05$). Sperm concentration varied numerically among ejaculation frequencies, ranging from $990.73 \pm 428.62 \times 10^6$ /mL at E2 to $2252.11 \pm 853.81 \times 10^6$ /mL at E1, but the differences were not statistically significant ($p > 0.05$). Total motility (TM), progressive motility (PM), fast motility (FM), slow motility (SM), circular motility (CM), and local motility (LM) were also comparable among ejaculation frequencies ($p > 0.05$). Similarly, the proportions of immotile

spermatozoa, sperm viability, sperm abnormalities, plasma membrane integrity (MI), and acrosome integrity (AI) did not differ significantly among ejaculation frequencies ($p > 0.05$). Thus, among the fresh semen quality variables evaluated, pH was the only parameter that differed significantly across ejaculation frequencies.

Sperm motility and kinematics

Sperm kinematic characteristics across ejaculation frequencies are presented in Table 2. Most kinematic parameters did not differ significantly among E1–E5 ($p > 0.05$). Significant differences were observed for distance average path (DAP) and ALH ($p < 0.05$). DAP was greater at E1 and E4 than at E2, whereas E3 and E5 had intermediate values and did not differ significantly from either group. For ALH was significantly lower at E5 than at E1–E4 ($p < 0.05$), with no significant differences among E1–E4. The remaining velocity-, distance-, trajectory-, and beat-related parameters did not differ significantly among ejaculation frequencies ($p > 0.05$).

Table 2: Kinematic parameters of progressively motile spermatozoa at different ejaculation frequencies in tropical Saanen bucks.

Kinematic parameter	E1	E2	E3	E4	E5
VCL ($\mu\text{m/s}$)	138.86 $\pm$ 52.48 ^a	133.28 $\pm$ 54.41 ^a	140.54 $\pm$ 57.38 ^a	156.28 $\pm$ 63.80 ^a	127.44 $\pm$ 52.03 ^a
VSL ($\mu\text{m/s}$)	109.08 $\pm$ 41.23 ^a	105.10 $\pm$ 42.91 ^a	92.66 $\pm$ 37.83 ^a	127.19 $\pm$ 51.93 ^a	103.61 $\pm$ 42.30 ^a
VAP ($\mu\text{m/s}$)	115.18 $\pm$ 43.53 ^a	110.65 $\pm$ 45.17 ^a	116.98 $\pm$ 47.76 ^a	133.89 $\pm$ 54.66 ^a	109.18 $\pm$ 44.57 ^a
DCL (μm)	52.53 $\pm$ 19.85 ^a	48.82 $\pm$ 19.93 ^a	51.13 $\pm$ 20.88 ^a	57.04 $\pm$ 23.28 ^a	48.28 $\pm$ 19.71 ^a
DSL (μm)	42.02 $\pm$ 15.88 ^a	39.55 $\pm$ 16.15 ^a	41.63 $\pm$ 16.99 ^a	47.06 $\pm$ 19.21 ^a	39.45 $\pm$ 16.11 ^a
DAP (μm)	51.53 $\pm$ 19.48 ^b	41.63 $\pm$ 16.99 ^a	43.66 $\pm$ 17.82 ^{ab}	49.64 $\pm$ 20.26 ^b	41.69 $\pm$ 17.02 ^{ab}
ALH (μm)	1.99 $\pm$ 0.75 ^b	1.97 $\pm$ 0.80 ^b	1.96 $\pm$ 0.80 ^b	2.05 $\pm$ 0.84 ^b	1.72 $\pm$ 0.70 ^a
BCF (Hz)	14.27 $\pm$ 5.39 ^a	12.40 $\pm$ 5.06 ^a	13.94 $\pm$ 5.69 ^a	14.24 $\pm$ 5.81 ^a	14.13 $\pm$ 5.77 ^a
HAC (rad)	0.22 $\pm$ 0.08 ^a	0.20 $\pm$ 0.08 ^a	0.20 $\pm$ 0.08 ^a	0.22 $\pm$ 0.09 ^a	0.20 $\pm$ 0.08 ^a
LIN (VSL/VCL)	0.76 $\pm$ 0.29 ^a	0.74 $\pm$ 0.30 ^a	0.77 $\pm$ 0.32 ^a	0.80 $\pm$ 0.33 ^a	0.93 $\pm$ 0.38 ^a
STR (VSL/VAP)	0.92 $\pm$ 0.35 ^a	0.92 $\pm$ 0.38 ^a	0.93 $\pm$ 0.38 ^a	0.78 $\pm$ 0.32 ^a	0.92 $\pm$ 0.38 ^a

DCL = Distance curved line; DSL = Distance straight line; DAP = Distance average path; HAC = Head angular change. Values are presented as mean $\pm$ SE. Different superscript letters within a row indicate significant differences among ejaculation frequencies based on pairwise comparisons with Holm adjustment ($p < 0.05$).

Seminal plasma protein molecular-weight fractions

The 1D-SDS-PAGE analysis identified five major seminal plasma protein fractions in the low- and medium-molecular-weight regions (Figure 1). Bands were identified at approximately 15, 20, 30, 35, and 50 kDa across the five ejaculation orders. The electrophoretic profiles showed differences in the detection and relative intensity of individual protein fractions among ejaculation orders.

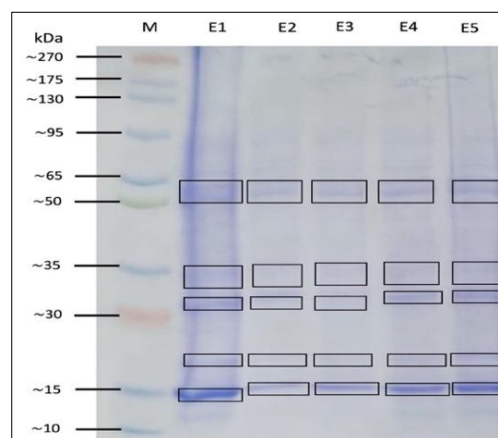


Figure 1: 1D-SDS-PAGE profiles of seminal plasma proteins in tropical Saanen bucks collected at different ejaculation orders (E1–E5). Major protein bands were identified at approximately 15, 20, 30, 35, and 50 kDa. Band intensity and detection varied among ejaculation orders. The approximately 15-kDa fraction showed the greatest mean relative abundance. M = prestained protein molecular-weight marker (10–270 kDa).

GelAnalyzer-assisted densitometric evaluation of the protein profiles is presented in Table 3. Across the detected fractions, the approximately 15-kDa fraction had the highest mean relative abundance (29.56 $\pm$ 4.69%), followed by the approximately 50-kDa (23.81 $\pm$ 5.75%) and 30-kDa (19.97 $\pm$ 4.12%) fractions. The approximately 20- and 35-kDa fractions had mean relative abundances of 14.12 $\pm$ 1.50% and 13.03 $\pm$ 2.45%, respectively. The

approximately 15-kDa fraction ranged from 25.10% at E1 to 35.87% at E5. The approximately 35- and 20-kDa fractions were not detected at E2 according to the densitometric data.

Table 3: Relative volume of seminal plasma protein fractions in tropical Saanen bucks at different ejaculation orders determined by 1D-SDS-PAGE and GelAnalyzer.

Protein fraction (kDa)	E1 (%)	E2 (%)	E3 (%)	E4 (%)	E5 (%)	Mean $\pm$ SD (%)
~50	15.88	29.18	28.74	24.04	21.22	23.81 $\pm$ 5.75
~35	14.69	—	12.69	15.08	9.68	13.03 $\pm$ 2.45
~30	19.08	24.88	13.85	21.72	20.33	19.97 $\pm$ 4.12
~20	16.31	—	13.66	13.61	12.90	14.12 $\pm$ 1.50
~15	25.10	30.23	31.07	25.55	35.87	29.56 $\pm$ 4.69

Relative abundance values were obtained from GelAnalyzer densitometric analysis and used to support visual evaluation of band intensity. Molecular weights were estimated relative to the prestained protein marker (10–270 kDa). Values are presented as relative abundance (%). Dash (—) indicates that the corresponding protein fraction was not detected.

Seminal plasma biochemical components

Seminal plasma biochemical values across ejaculation frequencies are presented in Table 4. Because samples from the six bucks were pooled within each ejaculation frequency, the values represent the corresponding pooled E1–E5 samples rather than individual buck means. Total protein decreased from 7.3 g/dL at E1 to 5.4 g/dL at E5, whereas albumin decreased from 3.2 g/dL at E1 and E2 to 2.3 g/dL at E5. GOT increased from 17.5 U/L at E1 to 21.3 U/L at E5. GPT increased from 21.5 U/L at E1 to 152.6 U/L at E3 and subsequently decreased to 111.3 U/L at E5. Cholesterol decreased from 79.6 mg/dL at E1 to 48.3 mg/dL at E5, whereas triglycerides decreased from 142.1 mg/dL to 56.7 mg/dL. Fructose fluctuated across ejaculation frequencies, with values of 25782, 15564, 11309, 25951, and 8854 μ g/mL at E1–E5, respectively.

Table 4: Seminal plasma biochemical components of tropical Saanen bucks at different ejaculation frequencies.

Biochemical variable	E1	E2	E3	E4	E5
Total protein (g/dL)	7.3	6.3	5.9	5.5	5.4
Albumin (g/dL)	3.2	3.2	2.7	2.6	2.3
GOT (U/L)	17.5	18.2	18.4	18.4	21.3
GPT (U/L)	21.5	45.4	152.6	137.6	111.3
Cholesterol (mg/dL)	79.6	66.3	52.9	50.8	48.3
Triglycerides (mg/dL)	142.1	114.5	81.2	59.6	56.7
Fructose (μ g/mL)	25782	15564	11309	25951	8854

Values represent measurements from pooled samples corresponding to each ejaculation frequency (E1–E5; n=5).

Correlations of ejaculation frequency with semen quality and seminal plasma biochemical components

Pearson's correlations between ejaculation frequency and semen quality parameters are presented in Figure 2. Semen color was negatively correlated with ejaculation frequency ($r = -0.488$, $p = 0.005$). In contrast, semen volume ($r = -0.330$, $p = 0.070$), pH ($r = -0.218$, $p = 0.239$), TM ($r = 0.115$, $p = 0.538$), PM ($r = 0.122$, $p = 0.515$), sperm viability ($r = -0.061$, $p = 0.744$), sperm abnormality ($r = 0.156$, $p = 0.402$), MI ($r = 0.153$, $p = 0.412$), and AI ($r = -0.198$, $p = 0.286$) were not significantly correlated with ejaculation frequency ($p > 0.05$).

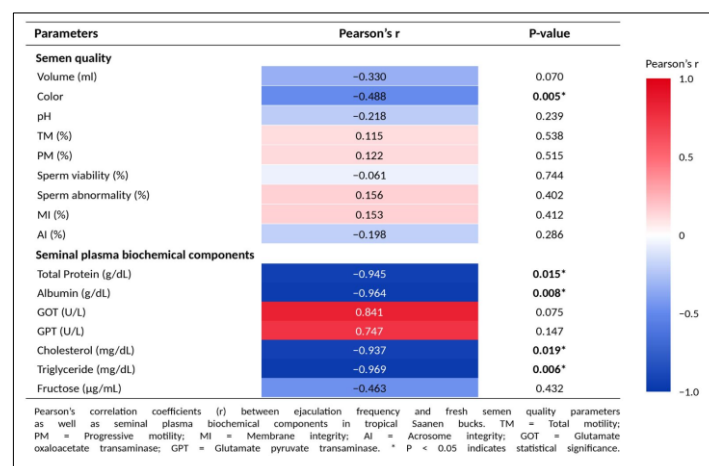


Figure 2: Pearson's correlation coefficients (r) between ejaculation frequency and fresh semen quality parameters in tropical Saanen bucks.

For the pooled seminal plasma biochemical observations, ejaculation frequency was negatively correlated with total protein ($r = -0.945$, $p = 0.015$), albumin ($r = -0.964$, $p = 0.008$), cholesterol ($r = -0.937$, $p = 0.019$), and triglycerides ($r = -0.969$, $p = 0.006$) (Figure 3). GOT ($r = 0.841$, $p = 0.075$), GPT ($r = 0.747$, $p = 0.147$), and fructose ($r = -0.463$, $p = 0.432$) were not significantly correlated with ejaculation frequency ($p > 0.05$). These biochemical correlations were calculated from the five pooled ejaculation frequency observations (E1–E5; $n = 5$).

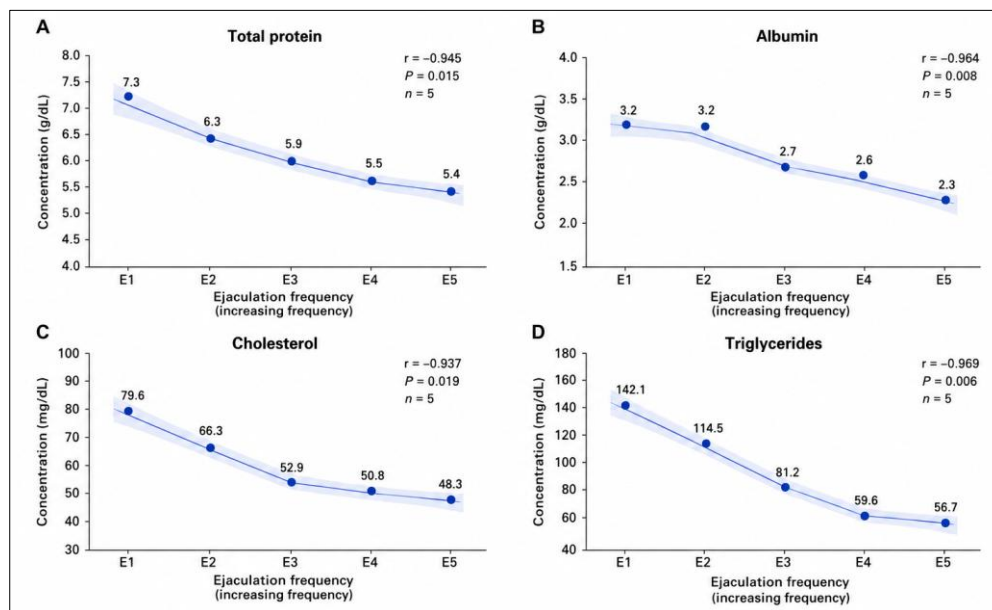


Figure 3: Linear relationships between ejaculation frequency and pooled seminal plasma biochemical parameters: (A) total protein, (B) albumin, (C) cholesterol, and (D) triglycerides. Each point represents the pooled value corresponding to one ejaculation frequency (E1–E5; $n = 5$). Pearson's correlation coefficient (r) and two-tailed p -value are shown in each panel.

DISCUSSION

Fresh semen quality at different ejaculation frequencies

The absence of significant differences in most macroscopic and microscopic semen parameters indicates that tropical Saanen bucks maintained most fresh semen quality characteristics across the evaluated ejaculation frequencies. These findings are consistent with previous studies reporting that increasing ejaculation frequency does not necessarily compromise overall semen quality [22]. Studies in small ruminants have also shown that semen quality traits such as motility, viability, and MI can remain relatively stable under varying physiological or management conditions, reflecting the capacity of the male reproductive system to maintain sperm function [23]. Such stability may indicate that the testes, epididymis, and accessory sex glands can maintain several functional characteristics during short-term repeated ejaculation.

In contrast, ejaculation frequency significantly affected semen pH, indicating that this parameter was more responsive to repeated ejaculation than most other conventional semen characteristics. Changes in pH may reflect alterations in the relative contribution or composition of accessory sex gland secretions during successive ejaculations. The magnitude of these changes may also be influenced by individual variation among bucks, including differences in physiological status, endocrine activity, metabolic characteristics, and reproductive capacity. Semen characteristics can vary among individuals because of genetic and physiological factors, even under similar environmental and management conditions [17, 18]. Such individual variability may contribute to differences in responses to repeated ejaculation. Despite the observed variation in pH, sperm functional parameters remained relatively stable. However, because this study evaluated fresh semen, we could not determine the consequences of changes in semen pH and seminal plasma composition for sperm resilience during storage or cryopreservation. Given the important role of seminal plasma in protecting spermatozoa against stresses associated with semen processing and preservation, further studies using preserved or cryopreserved semen are warranted.

Although semen volume showed a numerical decline with increasing ejaculation frequency, this difference was not statistically significant. Nevertheless, the numerical pattern may reflect incomplete replenishment of accessory sex gland secretions between successive ejaculations. Repeated ejaculation has been associated with reduced ejaculate volume and sperm output when the interval between collections is insufficient for complete

replenishment of seminal components [24]. Secretions from the accessory sex glands contribute proteins, ions, metabolites, and other constituents to seminal plasma. Therefore, short intervals between successive ejaculations could transiently affect the quantity or composition of these secretions. However, because semen volume did not decline significantly and collection intervals varied substantially among ejaculations, this interpretation should be considered cautiously.

Despite reduced seminal lipid concentrations across successive pooled ejaculation frequency samples, sperm motility, viability, MI, and AI remained largely unaffected. This observation is noteworthy because cholesterol and other membrane lipids contribute to sperm membrane stability, fluidity, and resistance to oxidative and physical stress [25]. The biochemical changes observed in the present study may not have been sufficient to cause detectable impairment of sperm function under fresh-semen evaluation conditions. Furthermore, semen was evaluated immediately after ejaculation and was not subjected to preservation or cryopreservation, during which membrane lipids have important roles in sperm resistance to cold shock, osmotic stress, and lipid peroxidation [25, 26]. Therefore, the biological consequences of reduced seminal plasma lipid concentrations may become more apparent during semen storage or cryopreservation and should be investigated under preservation conditions.

Although most conventional semen quality parameters remained unchanged, specific sperm kinematic characteristics varied among ejaculation frequencies. Kinematic analysis provides a more detailed characterization of sperm movement and may reveal physiological responses not readily detected with conventional semen evaluation alone [27, 28]. Because the biochemical environment surrounding spermatozoa, including ions, metabolites, proteins, and signaling molecules, can influence sperm movement, ejaculation-associated changes in seminal plasma composition may accompany changes in sperm movement characteristics. Overall, the present findings indicate that five successive ejaculations were associated with relatively stable conventional fresh semen quality, while semen pH, selected sperm kinematic parameters, and seminal plasma composition differed measurably. Integrating seminal plasma biochemistry with sperm kinematic assessment may therefore provide a more comprehensive characterization of reproductive responses to repeated semen collection [29, 30].

Sperm motility and kinematics

Differences in specific sperm kinematic characteristics were observed despite the overall stability of TM and PM across ejaculation frequencies. This finding indicates that repeated ejaculation was associated with changes in particular aspects of sperm movement rather than with a generalized reduction in the proportion of motile spermatozoa.

ATP-dependent flagellar activity drives sperm movement, which is regulated by mitochondrial energy metabolism and intracellular signaling pathways. The absence of significant differences in VCL, VSL, and VAP indicates that the principal velocity measurements remained relatively stable across ejaculation frequencies. In contrast, significant differences in ALH and DAP indicate changes in specific characteristics of lateral head displacement and sperm trajectory [27]. These findings suggest that selected kinematic measurements may respond differently to repeated ejaculation than conventional motility percentages.

At the molecular level, sperm kinematics can be regulated through pH- and Ca^{2+} -dependent signaling involving soluble adenylyl cyclase (sAC) [28]. Changes in seminal plasma composition associated with repeated ejaculation, particularly pH and protein content, could influence sAC-associated signaling and intracellular cyclic adenosine monophosphate (cAMP) production [pinto]. cAMP activates protein kinase A (PKA), which participates in phosphorylation events affecting axonemal and flagellar function. The sAC-cAMP-PKA pathway therefore provides a biologically plausible mechanistic context for variation in sperm movement characteristics [30]. However, the present study did not directly measure sAC activity, intracellular cAMP, PKA activity, or protein phosphorylation; consequently, this pathway cannot be established as the mechanism responsible for the observed differences in ALH and DAP [27].

Ca^{2+} influx through sperm-specific ion channels also contributes to regulation of flagellar bending and head oscillation [31, 32]. Sperm kinematics are known to be influenced by pH- and Ca^{2+} -dependent signaling involving sAC [28]. Changes in seminal plasma composition associated with repeated ejaculation may therefore interact with sAC activity and downstream cAMP-PKA signaling [29, 33]. Although these pathways were not directly evaluated in the present study, they provide a plausible mechanistic framework for future investigation of the observed alterations in ALH and DAP.

Overall, the findings demonstrate that ejaculation frequency was associated with differences in selected sperm kinematic characteristics while TM and PM remained stable. The present data cannot directly attribute the

observed changes to specific molecular signaling mechanisms. Nevertheless, CASA-derived kinematic variables, particularly ALH and DAP, may provide complementary functional information when assessing physiological responses to repeated ejaculation.

Seminal plasma protein molecular-weight fractions

Analysis of seminal plasma proteins using 1D-SDS-PAGE demonstrated major protein fractions within the low- and medium-molecular-weight regions in tropical Saanen bucks. Bands were identified at approximately 15, 20, 30, 35, and 50 kDa. However, according to the densitometric data in Table 3, the approximately 20- and 35-kDa fractions were not detected at E2. Therefore, the protein profile varied in both detection and relative abundance across ejaculation orders, rather than uniformly detecting every fraction at E1–E5.

GelAnalyzer densitometric analysis showed that the approximately 15-kDa fraction had the highest mean relative abundance among the detected protein fractions. Low-molecular-weight proteins therefore represented a substantial component of the seminal plasma protein profile. However, molecular-weight data alone do not identify the proteins represented by these bands or determine their specific biological functions.

Proteins within the approximately 15–20-kDa range have frequently been associated with binders of sperm proteins/fibronectin type II domain-related seminal plasma proteins in domestic ruminants [25, 34, 35]. Proteins in these families can interact with sperm membrane phospholipids, participate in membrane remodeling, and help regulate capacitation-related events [25]. Accordingly, the predominance of an approximately 15-kDa fraction provides an appropriate basis for further investigation of low-molecular-weight seminal plasma proteins in Saanen bucks. Nevertheless, identification of the approximately 15-kDa band as a specific protein or protein family would require proteomic confirmation.

Similarly, proteins within the approximately 30–35-kDa molecular-weight region may include proteins involved in membrane organization, cellular protection, or regulation of the seminal plasma environment [36]. Proteins within the approximately 50-kDa region may also include regulatory proteins or glycoproteins involved in extracellular stabilization, ion homeostasis, and sperm protection [37–40]. However, because this study used molecular-weight separation rather than protein identification, these functional associations should be treated as possible biological contexts rather than identities assigned to the observed bands.

The 1D-SDS-PAGE findings can also be considered alongside the CASA-derived kinematic responses. Significant differences were observed in ALH and DAP, despite no significant differences in TM and PM. Seminal plasma proteins can influence sperm function through interactions with sperm membranes, ion transport, and intracellular signaling processes [29, 41]. Consequently, variation in seminal plasma protein composition could potentially accompany changes in sperm movement characteristics [36]. However, this study found no statistically significant correlation between individual protein fractions and kinematic parameters, so a causal relationship cannot be established.

Ejaculation-associated variation in seminal plasma proteins may also be relevant to Ca^{2+} -dependent signaling involved in sperm movement. The sAC-cAMP-PKA pathway and sperm-specific CatSper channels regulate intracellular Ca^{2+} signaling, axonemal phosphorylation, and sperm motility [37, 42]. Evidence from buck spermatozoa further supports the importance of CatSper-mediated Ca^{2+} regulation for sperm kinematics and reproductive function [43]. These mechanisms provide a possible biological framework for investigating the differences observed in ALH and DAP [40]. Nevertheless, the present study did not measure intracellular Ca^{2+} concentrations, CatSper activity, ATP production, or associated signaling pathways; these mechanistic relationships therefore require direct experimental validation.

Collectively, the findings indicate that successive ejaculation was accompanied by variation in the relative abundance and detection of seminal plasma protein molecular-weight fractions. The approximately 15-kDa fraction remained predominant by mean relative abundance, whereas the other molecular-weight fractions showed varying relative abundances across ejaculation orders. Seminal plasma proteins may contribute to sperm function and regulate sperm kinematics [27, 44], but identifying individual proteins and directly evaluating their relationships with sperm function will require quantitative proteomic and functional studies.

Seminal plasma biochemical components

Pooled seminal plasma samples showed marked changes in biochemical composition across successive ejaculation frequencies. Total protein and albumin progressively decreased from E1 to E5, suggesting reduced availability of these seminal plasma constituents during repeated ejaculation [45]. Seminal plasma proteins originate primarily from secretions of the reproductive tract and perform diverse biological functions. These

include molecular chaperone activity [41], antioxidant functions [46], and modulation of sperm membrane stability [47, 48]. Repeated ejaculation could therefore alter the biochemical environment surrounding spermatozoa through changes in the contribution of these secretions. Reduced availability of seminal plasma proteins may also alter the protein coating surrounding spermatozoa [25] and potentially influence their susceptibility to mechanical or oxidative stress [34]. However, the present study did not directly measure oxidative damage, and this possibility requires experimental confirmation.

Albumin is involved in cholesterol transport and participates in cholesterol efflux from the sperm plasma membrane during capacitation-associated membrane remodeling [49]. The progressive decrease in albumin across the pooled ejaculation frequency samples could therefore have functional relevance to the seminal environment. Albumin-mediated cholesterol removal is associated with membrane remodeling, Ca^{2+} influx, and cAMP-dependent PKA signaling [25, 47]. Nevertheless, capacitation, cholesterol efflux, intracellular Ca^{2+} , and PKA activity were not measured in the present study. Therefore, the observed decrease in albumin cannot be interpreted as direct evidence of altered capacitation-related signaling.

GPT activity increased substantially from E1 to E3 and subsequently decreased at E4 and E5. This pattern may provide information regarding changes in the metabolic composition of seminal plasma during repeated ejaculation [50]. GPT participates in amino acid metabolism and alanine-glutamate transamination [51], processes that are linked to cellular intermediary metabolism and the tricarboxylic acid cycle [52]. Under some physiological or pathological conditions, increased extracellular transaminase activity can be associated with changes in membrane permeability or cellular leakage [53]. Transaminase activity may also relate to metabolic processes that support cellular energy requirements [54]. However, this study did not determine the cellular source of the increased GPT activity, and the observed pattern cannot be attributed specifically to metabolic exhaustion, sperm membrane damage, or accessory gland cell injury.

In contrast, GOT activity increased only modestly across ejaculation frequencies. GOT participates in amino acid metabolism and is associated with the malate–aspartate shuttle, which contributes to transfer of reducing equivalents associated with mitochondrial oxidative metabolism [55]. The relatively limited variation in GOT occurred concurrently with stable conventional sperm motility parameters [56]. Nevertheless, this observation does not demonstrate preserved mitochondrial function because mitochondrial activity was not measured directly. Similarly, although altered GOT activity has been discussed in relation to cellular or mitochondrial stress [57], the numerical increase observed at later ejaculation orders cannot be interpreted as evidence of mitochondrial stress without direct mitochondrial measurements.

Cholesterol and triglyceride concentrations decreased progressively across the pooled ejaculation frequency samples, demonstrating substantial changes in seminal plasma lipid composition [58]. Cholesterol is an important structural component of sperm membranes and helps regulate membrane organization and capacitation-associated processes [59]. Reduced cholesterol availability can potentially influence membrane properties, ion permeability, and associated signaling processes [60]. Triglycerides can provide fatty acids that participate in cellular energy metabolism and β -oxidation [61]. Accordingly, reductions in triglycerides could theoretically alter the availability of lipid-derived metabolic substrates. However, ATP production and lipid utilization were not measured; therefore, the observed decline in triglycerides cannot be interpreted as direct evidence of reduced ATP generation [58] or as the cause of changes in sperm velocity or trajectory [29].

Integration of the biochemical findings with the 1D-SDS-PAGE molecular-weight profiles indicates that repeated ejaculation was accompanied by changes in multiple components of seminal plasma. Variations in protein fractions and biochemical constituents may biologically relate to sperm membrane interactions, signaling, and metabolism. Potential pathways include Ca^{2+} signaling and cAMP-PKA-associated processes [25], as well as mitochondrial oxidative metabolism [46]. Such pathways could, in principle, be relevant to sperm kinematic behavior [27]. However, these molecular mechanisms were not directly investigated, and their relationship to the observed biochemical and kinematic patterns remains experimentally unestablished.

Correlations of ejaculation frequency with fresh semen quality and seminal plasma biochemical components

The correlation analysis indicated that ejaculation frequency was more consistently associated with changes in selected seminal plasma biochemical components than with conventional fresh semen quality parameters. Most conventional semen quality characteristics, including TM, PM, sperm viability, MI, and AI, were not significantly correlated with ejaculation frequency. This finding is consistent with the repeated-measures results showing relative stability of these sperm characteristics across successive ejaculations.

The absence of significant correlations between ejaculation frequency and TM or PM indicates that the

proportions of motile and progressively motile spermatozoa did not show a significant linear association with increasing ejaculation order. Sperm motility depends on ATP availability [62] and is supported by mitochondrial oxidative phosphorylation and glycolytic pathways [63]. The observed maintenance of motility is therefore consistent with preserving the cellular processes necessary to sustain movement [64]. However, because ATP concentrations and metabolic fluxes were not directly measured, the present results cannot establish that these specific energy pathways remained unchanged.

Similarly, the lack of significant associations with sperm viability, MI, and AI suggests that increasing ejaculation frequency was not accompanied by a detectable linear decline in these cellular characteristics. Seminal plasma contains components that can help maintain sperm membranes and protect spermatozoa, but the specific protective mechanisms behind the observed stability were not directly evaluated.

In contrast, semen color showed a moderate negative correlation with ejaculation frequency ($r = -0.488$, $p = 0.005$). Semen color can be influenced by ejaculate composition, including sperm and seminal plasma constituents. The observed association occurred alongside changes in several pooled seminal plasma biochemical variables. However, the present analyses do not establish that depletion of any particular protein or lipid component caused the change in semen color. Therefore, semen color should be interpreted as an observed phenotypic correlate of ejaculation frequency rather than a validated indirect biomarker of seminal plasma composition.

The strongest biochemical relationships involved total protein, albumin, cholesterol, and triglycerides, all of which showed negative correlations with ejaculation frequency. These findings are consistent with their progressive numerical decreases from E1 to E5 and may reflect incomplete replenishment of reproductive tract secretions between successive ejaculations [65]. Seminal plasma proteins and lipids participate in sperm membrane interactions [25], ion regulation, and metabolic support [66]. Therefore, changes in these constituents could modify the extracellular environment surrounding spermatozoa even when conventional measures of sperm viability and motility remain relatively stable.

Albumin showed a particularly strong negative correlation with ejaculation frequency. Albumin participates in cholesterol transport and membrane remodeling associated with sperm capacitation [67]. Consequently, changes in albumin concentration could potentially influence the balance of sperm membrane remodeling processes and downstream functional characteristics [68]. However, capacitation status was not directly assessed, and the observed correlation does not demonstrate a causal effect of albumin depletion on sperm function. Similarly, declining cholesterol concentrations could influence membrane organization and signaling processes involved in regulating sperm function [69], but these mechanisms were not directly evaluated.

Triglycerides also showed a strong negative correlation with ejaculation frequency, indicating progressively lower triglyceride concentrations across the pooled samples. Although lipid-derived substrates can contribute to cellular metabolism, the present study did not measure sperm lipid utilization or ATP generation. Therefore, the decrease in seminal plasma triglycerides should not be interpreted as direct evidence of reduced sperm energy availability. The concurrent stability of conventional motility percentages and variation in selected kinematic parameters warrants further investigation using direct metabolic and functional measurements.

GOT and GPT showed positive but nonsignificant correlations with ejaculation frequency. These patterns may reflect variation in the metabolic or cellular composition of seminal plasma during repeated ejaculation, but the lack of statistical significance prevents us from concluding that either enzyme increased linearly with ejaculation frequency. Changes in extracellular enzyme activity can occur with cellular turnover or altered membrane permeability [53], but the present study did not directly assess these mechanisms.

Fructose, an important carbohydrate component of seminal plasma and a potential energy substrate for spermatozoa [70, 71], showed a negative but nonsignificant correlation with ejaculation frequency ($r = -0.463$, $p = 0.432$). Its values fluctuated across E1–E5 rather than showing a consistent monotonic response. Consequently, the present data do not support either a significant positive or negative relationship between ejaculation frequency and seminal plasma fructose concentration.

The biochemical changes observed during repeated ejaculation may also interact with mechanisms that protect spermatozoa from oxidative stress [44]. However, the present study did not report total antioxidant capacity or direct oxidative stress markers among the evaluated biochemical variables. Therefore, no conclusion can be drawn regarding an ejaculation-dependent antioxidant response.

Taken together, the correlation analysis indicates that ejaculation frequency was associated more consistently with changes in selected pooled seminal plasma biochemical variables than with conventional fresh

semen quality parameters. Biochemical profiling may therefore provide complementary information regarding changes occurring across successive ejaculations. Nevertheless, an important distinction exists between the datasets: correlations involving fresh semen quality were derived from individual ejaculate-level observations, whereas correlations involving seminal plasma biochemical components were calculated from only five pooled ejaculation frequency observations (E1–E5; $n = 5$). The biochemical correlations should therefore be interpreted cautiously as frequency-level associations and not as independent buck-level relationships.

Study considerations

Several limitations should be considered when interpreting the present findings. First, the study included only six Saanen bucks, which may limit the generalizability of the findings to broader populations, breeds, or management conditions. Second, the intervals between successive ejaculations varied substantially, ranging from 5 to 373 min. This variation could have influenced replenishment of seminal plasma constituents independently of ejaculation order and should be considered when interpreting ejaculation-associated changes.

Third, the biochemical and 1D-SDS-PAGE analyses were performed using samples pooled across the six bucks within each ejaculation frequency. Consequently, individual buck variability could not be evaluated for these outcomes. The biochemical correlations were therefore based on only five pooled observations, while duplicate biochemical measurements represented technical rather than independent biological replication.

Finally, although seminal plasma biochemical characteristics and protein molecular-weight profiles changed with increasing ejaculation frequency, mass spectrometry did not identify individual proteins, so their specific functional roles in sperm physiology could not be established. Similarly, the molecular mechanisms discussed in relation to sperm kinematics, including Ca^{2+} signaling, sAC-cAMP-PKA signaling, CatSper activity, ATP production, and mitochondrial metabolism, were not directly evaluated. Future studies involving larger populations, standardized ejaculation intervals, individual seminal plasma analyses, quantitative proteomics, direct molecular measurements, and functional validation are required to elucidate the mechanisms underlying these responses.

CONCLUSION

Repeated ejaculation across five successive collections produced limited changes in conventional fresh semen quality in tropical Saanen bucks. Most macroscopic and microscopic semen characteristics, including semen volume, sperm concentration, TM, PM, sperm viability, sperm abnormality, MI, and AI, remained statistically unchanged, whereas semen pH differed significantly among ejaculation frequencies. Among sperm kinematic characteristics, DAP and ALH differed significantly, while the other evaluated kinematic parameters remained relatively stable. The 1D-SDS-PAGE analysis demonstrated variation in the relative abundance and detection of seminal plasma protein molecular-weight fractions, with the approximately 15-kDa fraction showing the highest mean relative abundance. In pooled seminal plasma samples, total protein, albumin, cholesterol, and triglycerides decreased with successive ejaculations and showed strong negative correlations with ejaculation frequency.

The maintenance of most conventional fresh semen quality parameters across five successive ejaculations suggests that tropical Saanen bucks can maintain several important semen characteristics during short-term repeated semen collection. However, the concurrent changes in semen pH, selected sperm kinematic characteristics, seminal plasma protein fractions, and biochemical components indicate that conventional semen evaluation alone may not fully characterize the physiological responses associated with repeated ejaculation. Integrating CASA-derived sperm kinematics with seminal plasma biochemical and protein profiling may therefore provide complementary information for evaluating bucks subjected to intensive mating or semen collection.

A major strength of this study was the integrated assessment of conventional fresh semen quality, CASA-derived sperm kinematics, seminal plasma biochemical components, and protein molecular-weight profiles within the same repeated ejaculation model. The collection of five successive ejaculates from each buck enabled characterization of progressive responses across ejaculation orders. The combination of functional sperm measurements with biochemical and 1D-SDS-PAGE analyses provided a broader assessment of reproductive responses than conventional semen evaluation alone.

The study included only six Saanen bucks, which limits the generalizability of the findings to larger populations and other environmental or management conditions. The intervals between successive ejaculations varied substantially (5–373 min), potentially influencing seminal plasma replenishment independently of ejaculation order. In addition, biochemical and 1D-SDS-PAGE analyses were performed using samples pooled across the six bucks within each ejaculation frequency, preventing evaluation of individual buck variability.

Consequently, biochemical correlations were based on only five pooled observations (E1–E5; $n = 5$) and should be interpreted as frequency-level rather than buck-level associations. Furthermore, 1D-SDS-PAGE characterized protein molecular-weight fractions but did not identify individual proteins, and the molecular signaling pathways potentially associated with the observed sperm kinematic changes were not directly evaluated.

Future studies should include more bucks, standardized intervals between successive ejaculations, and individual rather than pooled seminal plasma samples to provide adequate biological replication. Quantitative proteomic approaches, including mass spectrometry, are needed to identify the proteins represented by the observed molecular-weight fractions and determine their functional relationships with sperm characteristics. Further investigations should also directly evaluate intracellular Ca^{2+} signaling, sAC-cAMP-PKA pathways, CatSper activity, energy metabolism, oxidative status, and other mechanisms potentially associated with changes in sperm kinematics. Studies incorporating semen preservation, cryopreservation, fertility outcomes, and repeated mating under field conditions would further clarify the biological and practical consequences of intensive ejaculation frequency in tropical Saanen bucks.

Within the conditions of this study, five successive ejaculations were associated with relatively stable conventional fresh semen quality but measurable changes in semen pH, selected sperm kinematic characteristics, seminal plasma biochemical composition, and protein molecular-weight profiles. These findings indicate that the effects of repeated ejaculation may be more readily reflected in selected seminal plasma and kinematic variables than in conventional semen quality measurements. The results provide a basis for further investigation of ejaculation frequency management in tropical Saanen bucks, while the limited number of animals, variable collection intervals, and pooled seminal plasma analyses should be considered when extrapolating the findings to breeding or semen collection programs.

DATA AVAILABILITY

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

GENERATIVE AI DECLARATION

Generative artificial intelligence (AI) tools were used exclusively to enhance the language, grammar, and readability of the manuscript. All scientific content, data analyses, interpretation of results, and conclusions were critically reviewed and validated by the authors. The authors assume full responsibility for the accuracy, integrity, and originality of the work, and no AI tool is credited as an author.

AUTHORS' CONTRIBUTIONS

AR, RIA, NWKK, and AB designed the study, conducted semen collection and laboratory analyses, data analysis, and drafted the manuscript. RIA, NWKK, SS, and MFU supervised the research design and data interpretation and critically revised the manuscript. AR, RIA, SS, MFU, RP, and AB contributed to study conceptualization, protein analysis, data interpretation, and manuscript revision. All authors have read and approved the final manuscript.

ACKNOWLEDGMENTS

The authors acknowledge financial support from IPB University through the College Endowment Funds DAPT EQUITY-QS By Subject (Agreement No. 13266/IT3.D10/PT.01.03/P/B/2026) and the Research Center for Applied Zoology, National Research and Innovation Agency (BRIN), Indonesia.

COMPETING INTERESTS

The authors declare that they have no competing interests.

PUBLISHER'S NOTE

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