CYTOGENETIC BIOMARKERS OF REPRODUCTIVE AGING: TELOMERE LENGTH AND CHROMATIN INTEGRITY IN SPERMATOZOA AND LEUKOCYTES OF FARMED DEER

Abstract

Telomeres play a critical role in maintaining chromosomal integrity and are increasingly recognized as indicators of biological aging and reproductive competence in mammals. However, information on telomere dynamics in cervids remains scarce. This study examined leukocyte telomere length (LTL) and sperm telomere length (STL) as markers of reproductive aging in three deer species.

Blood and semen samples were obtained from twenty-seven breeding stags (nine per species) aged 3–11 years. Telomere length was quantified using quantitative PCR and expressed as T/S ratios. Semen was evaluated for volume, sperm concentration, motility, kinematic characteristics, and chromatin condensation, while oxidative status was assessed using d-ROMs and biological antioxidant potential assays. Associations among telomere length, age, semen characteristics, oxidative markers, and species were analyzed using nonparametric tests, multivariate regression, and principal component analysis.

Leukocyte and sperm telomere lengths were strongly correlated ($\rho = 0.94$, $p < 0.001$). Both parameters showed a marked age-related decline, with reductions in STL occurring earlier than those in LTL. Sperm telomere length exhibited strong positive associations with semen volume, sperm concentration, and chromatin condensation (all $p < 0.001$). Significant interspecies differences were detected for STL and semen volume, whereas systemic oxidative stress indicators were not associated with telomere length. Multivariate analysis identified STL as the most influential predictor of semen quality.

These findings indicate that sperm telomere length is a sensitive indicator of reproductive aging in deer and may provide a valuable tool for reproductive management and conservation strategies.

Keywords: telomere length, reproductive aging, deer, semen quality, chromatin condensation, reproductive biomarker

INTRODUCTION

Reproductive aging presents a significant challenge in wildlife conservation and captive breeding programs, particularly for cervid species with economic and ecological importance [1]. In deer farming, which has expanded globally for meat, antler, and breeding stock production, age-related decline in male fertility can limit productivity and genetic diversity [2]. However, reliable biomarkers for assessing reproductive aging in deer remain limited, with current methods

focusing primarily on conventional semen parameters that may not capture early functional decline [3].

Telomeres, repetitive nucleotide sequences at chromosome ends, protect genomic integrity and shorten with cellular replication and oxidative damage [4]. Telomere length (TL) has been shown to be an important biomarker of biological aging in various mammalian species, with associations demonstrated between shortened telomeres and reduced fertility, increased disease susceptibility, and decreased lifespan [5]. In humans and domestic animals, sperm telomere length (STL) specifically correlates with semen quality parameters, including concentration, motility, and DNA integrity [6,7]. Leukocyte telomere length (LTL), while more accessible, may not directly reflect germline aging but can provide systemic aging indicators [8].

Some animal models have provided particular insights into telomere biology relevant to domestic and wild animals, demonstrating strong correlations between STL, age, and semen quality [9]. However, comparative studies in cervids are notably lacking, despite the phylogenetic proximity and similar reproductive challenges faced by deer breeding programs. Deer species exhibit diverse life histories, reproductive strategies, and longevity patterns, offering a valuable comparative framework for understanding telomere dynamics in relation to aging and fertility [10].

Three deer species with distinct characteristics are commonly reared in captivity: Axis axis (chital deer), a smaller, shorter-lived species adapted to tropical environments; Rusa timorensis (Javan deer), an intermediate species with good reproductive performance; and Rusa unicolor (sambar deer), a larger, longer-lived species with greater semen production but potentially different aging patterns [11]. These species differences provide an opportunity to investigate how life history traits influence telomere maintenance and reproductive aging.

This study aimed to: (1) establish baseline LTL and STL values for three deer species using quantitative PCR; (2) evaluate correlations between TL, age, and semen quality parameters; (3) assess breed differences in TL dynamics; and (4) explore relationships between TL and systemic oxidative stress. We hypothesized that STL would show stronger associations with reproductive parameters than LTL, decline earlier with age, and exhibit breed-specific patterns reflecting different life history strategies. The findings contribute to understanding telomere biology in cervids and may inform the development of TL as a practical biomarker for reproductive assessment in deer breeding and conservation programs.

MATERIALS AND METHODS

Study Design and Animals

This cross-sectional study was conducted at the PTH Lenggong Deer Breeding Center, Perak, Malaysia. A total of 27 clinically healthy breeding stags were included, comprising nine animals from each of three species: Axis axis (chital deer), Rusa timorensis (Javan deer), and Rusa unicolor (sambar deer). Animals ranged in age from 3 to 11 years (mean ± SD: 7.0 ± 2.5 years) and were maintained under standardized nutritional and environmental conditions. Inclusion criteria required proven fertility and absence of clinical illness or recent medication. The study protocol was approved by the Animal Ethics Committee of Universiti Putra Malaysia (Reference: UPM/IACUC/AUP-RO47/2017).

Sample Collection

Five milliliters of venous blood were collected via jugular venipuncture using sterile 21-gauge needles. Samples were divided between EDTA tubes (for leukocyte DNA extraction) and clot-

activator tubes (for serum oxidative stress analysis). Serum was separated by centrifugation at 3,000 × g for 10 minutes and stored at -80°C until analysis.

Semen was collected by electroejaculation under sedation with xylazine hydrochloride (0.2 mg/kg) and ketamine (2.0 mg/kg). Only the sperm-rich fraction was collected to avoid prostatic fluid contamination. Samples were immediately evaluated for macroscopic parameters and divided into aliquots for microscopic analysis and cryopreservation.

Semen Evaluation

Semen volume was measured using graduated tubes. Concentration was determined using a Neubauer hemocytometer. Computer-assisted sperm analysis (CASA; Sperm Class Analyzer, Microptic SL, Barcelona, Spain) was performed to assess motility parameters: total motility (%), progressive motility (%), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity (LIN, %), straightness (STR, %), wobble (WOB, %), beat-cross frequency (BCF, Hz), and amplitude of lateral head displacement (ALH, μm). A minimum of 200 spermatozoa were analyzed per sample at 60 frames per second.

Sperm Chromatin Condensation Assessment

Chromatin condensation was evaluated using Diff-Quik staining (BioOptica, Milan, Italy). Briefly, 10 μL of raw semen was smeared on glass slides, air-dried, fixed in methanol (10 seconds), stained sequentially in eosin (5 seconds) and thiazine (10 seconds), rinsed with distilled water, and air-dried. Slides were examined under a Leica DM750 light microscope at 1000× magnification under oil immersion. Two hundred spermatozoa per sample were classified as: normally condensed (light-stained, homogeneous chromatin), partially decondensed (moderately stained, granular appearance), or fully decondensed (dark-stained, irregular

chromatin). The condensation index was calculated as percentage of normally condensed spermatozoa.

Telomere Length Measurement by Quantitative PCR

Genomic DNA was extracted from leukocytes and spermatozoa using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA). DNA concentration and purity were assessed by spectrophotometry (NanoDrop 1000; Thermo Fisher Scientific), with samples requiring A260/A280 > 1.7 and A260/A230 > 1.8 for inclusion.

Relative telomere length (RTL) was measured by quantitative PCR (qPCR) using a modified Cawthon method. Reactions were performed in triplicate on a CFX96 Touch Real-Time PCR System (Bio-Rad, Hercules, CA, USA) in 10 µL volumes containing 30 ng DNA, 1× iTaq Universal SYBR Green Supermix (Bio-Rad), and 0.3 µM of each primer. Telomere (T) primers were: TelomereA (Forward): 5′CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′ and Telomere B (Reverse): 5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3′. The single-copy reference gene GAPDH (S) primers were: Forward: 5'-GTCGGTTTGTGGATCTCTCT-3' and Reverse: 5'-GGAGATGATGACCCGTTT-3'. Cycling conditions: 95°C for 3 minutes; 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Melt curve analysis confirmed primer specificity. The T/S ratio was calculated using the ΔΔCt method with GAPDH normalization. Amplification efficiencies for T and S primers were 98.3% and 98.6%, respectively, with inter-assay coefficient of variation <5%. Results are expressed as relative T/S ratios for leukocyte TL (LTL) and sperm TL (STL).

Oxidative Stress Assessment

Systemic oxidative stress was evaluated using serum reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) tests (Diacron International, Grosseto, Italy). The d-ROMs test measures hydroperoxide-derived radicals via colorimetric reaction at 505 nm, with results expressed in Carratelli units (U CARR). The BAP test assesses ferric ion reduction capacity, with results expressed in μmol/L. Analyses were performed on a SAT450 analyzer (KPM Analytics, Marlborough, MA, USA) according to manufacturer protocols.

Statistical Analysis

Data normality was assessed using Shapiro-Wilk tests. Non-parametric tests were applied due to non-normal distributions and small sample size. Spearman's rank correlation coefficients (ρ) were calculated to assess relationships between variables, with interpretation: $\rho = 1.0$ (complete), 0.70-0.99 (strong), 0.50-0.69 (moderate), 0.01-0.49 (weak). Mann-Whitney U tests compared age groups, and Kruskal-Wallis tests with post-hoc Dunn's tests assessed breed differences. Multivariate linear regression analyzed predictors of semen quality. Principal component analysis (PCA) explored multivariate patterns. Statistical significance was set at $p < 0.05$. Analyses were performed using SPSS v26 (IBM Corp., Armonk, NY, USA) and Python 3.9 with SciPy, scikit-learn, and pandas libraries.

RESULTS

Descriptive Statistics

The study included 27 breeding stags equally distributed among three deer species (Table 1). Mean age was 7.0 ± 2.5 years (range: 3-11 years). Mean leukocyte telomere length (LTL) was 0.68 ± 0.10 T/S ratio, while mean sperm telomere length (STL) was 0.86 ± 0.14 T/S ratio. Breed-specific differences were observed: Axis axis exhibited the highest STL (0.87 ± 0.16) but lowest semen volume (2.1 ± 0.7 ml), whereas Rusa unicolor had the lowest STL (0.84 ± 0.11) but

highest semen volume (4.1 ± 0.7 ml). Chromatin condensation ranged from 75-94% (mean: 86.2 ± 5.8%), with Rusa unicolor showing the highest values (87.8 ± 5.2%).

Table 1. Descriptive statistics by deer species

Parameter	*Axis axis* (n=9)	*Rusa timorensis* (n=9)	*Rusa unicolor* (n=9)	Overall (n=27)
Age (years)	7.00 ± 2.70	7.00 ± 2.70	7.00 ± 2.70	7.00 ± 2.50
LTL (T/S ratio)	0.71 ± 0.10	0.68 ± 0.09	0.65 ± 0.09	0.68 ± 0.10
STL (T/S ratio)	0.87 ± 0.16	0.87 ± 0.13	0.84 ± 0.11	0.86 ± 0.14
Semen volume (ml)	2.10 ± 0.70	3.10 ± 0.70	4.10 ± 0.70	3.10 ± 1.00
Sperm concentration ($\times 10^6$/ml)	377.00 ± 65.00	436.00 ± 76.00	526.00 ± 70.00	446.00 ± 86.00
Chromatin condensation (%)	85.00 ± 6.40	85.90 ± 5.90	87.80 ± 5.20	86.2 ± 5.8
Total motility (%)	66.80 ± 7.30	71.00 ± 6.70	73.00 ± 6.60	70.30 ± 7.00
d-ROMs (U CARR)	74.80 ± 7.00	69.00 ± 6.60	64.00 ± 6.00	69.30 ± 7.60
BAP (µmol/L)	2114.00 ± 97.00	2184.00 ± 89.00	2262.00 ± 83.00	2187.00 ± 106.00

3.2. Telomere Length Correlations

A strong positive correlation was observed between LTL and STL ($\rho = 0.94$, $p < 0.001$; Fig. 1A). Both LTL and STL showed strong negative correlations with age (LTL: $\rho = -0.97$, $p < 0.001$; STL: $\rho = -0.97$, $p < 0.001$; Fig. 1B). STL was positively correlated with semen volume ($\rho = 0.99$, $p < 0.001$; Fig. 1C) and sperm concentration ($\rho = 0.96$, $p < 0.001$), and strongly correlated with chromatin condensation percentage ($\rho = 0.99$, $p < 0.001$; Fig. 1D). LTL showed a moderate positive correlation with sperm concentration ($\rho = 0.96$, $p < 0.001$). No significant correlationswere found between telomere lengths and systemic oxidative stress markers (d-ROMs and BAP; $p > 0.05$ for all comparisons).

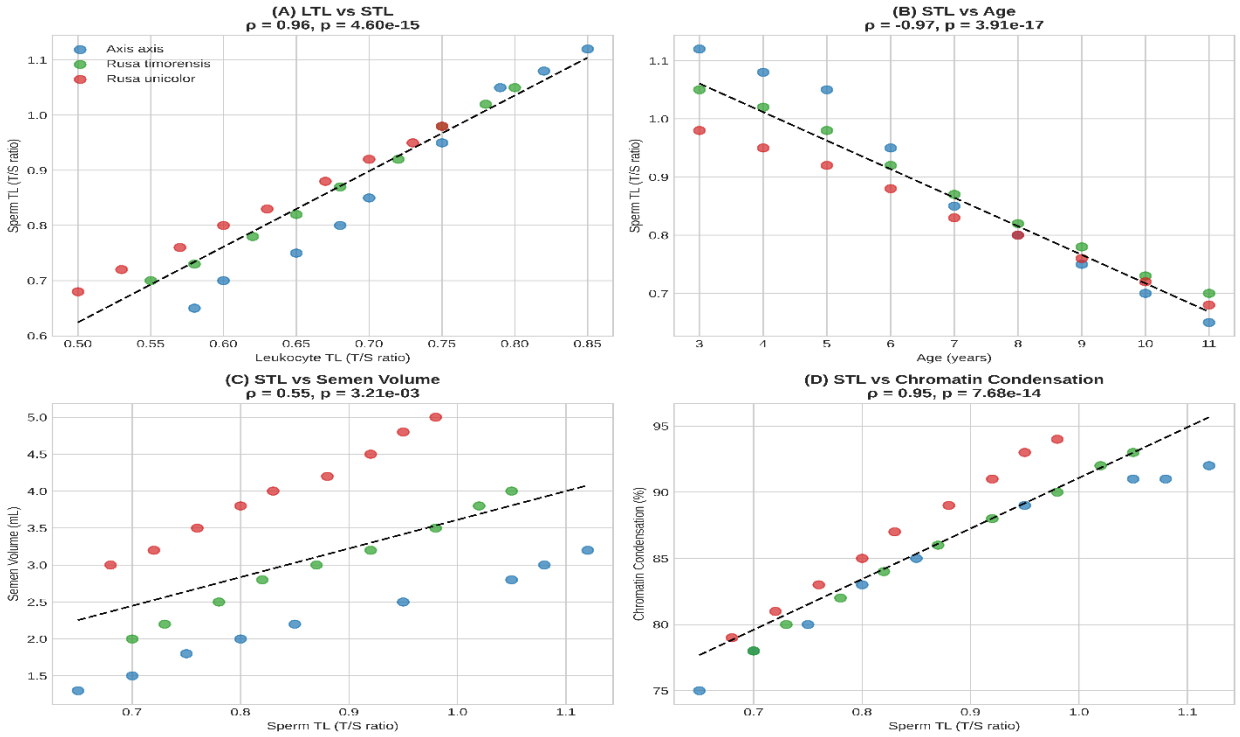

FIGURE 1: Four scatterplots showing: (A) LTL vs STL, (B) STL vs Age, (C) STL vs Semen Volume, (D) STL vs Chromatin Condensation. Points colored by breed (blue: Axis axis, green: Rusa timorensis, red: Rusa unicolor). Dashed black lines show linear regression fits. Spearman's ρ and p-values shown in titles.

3.3. Age Group Comparisons

Significant age-related declines in telomere length were observed at species-specific thresholds (Fig. 2). For STL, animals ≤6 years (n=12) showed significantly higher values (1.01 ± 0.07 T/S ratio) compared to those >6 years (n=15; 0.74 ± 0.08 T/S ratio; $U = 180$, $p < 0.001$, effect size $r = 0.87$). For LTL, the critical threshold was 7.5 years, with animals ≤7.5 years (n=15) exhibiting higher LTL (0.76 ± 0.06 T/S ratio) than those >7.5 years (n=12; 0.59 ± 0.08 T/S ratio; $U = 180$, $p < 0.001$, effect size $r = 0.87$).

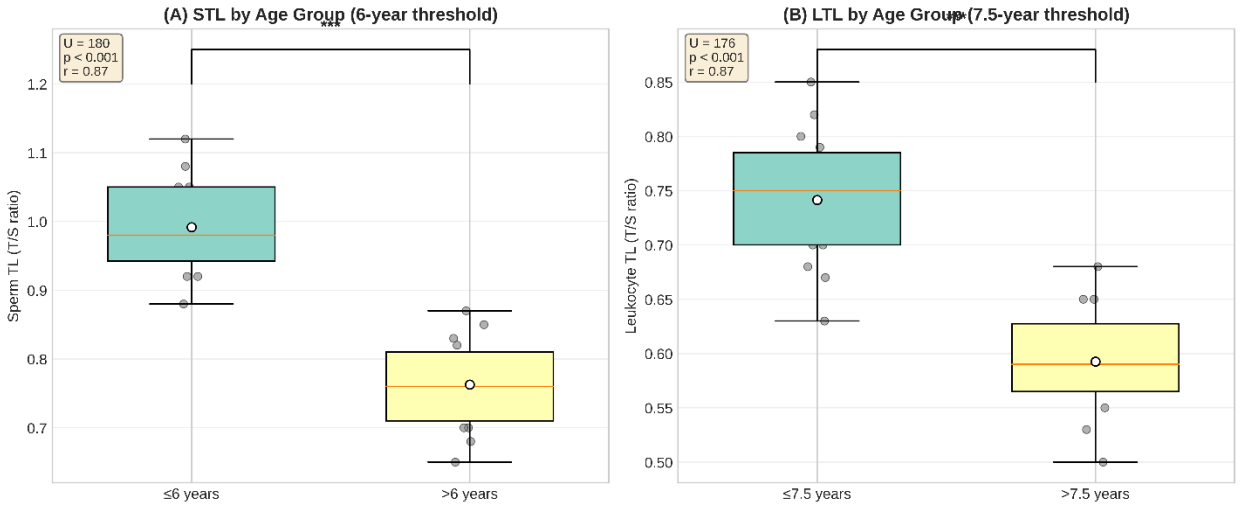

FIGURE 2: Dual boxplot figure comparing (A) STL in animals ≤6 vs >6 years, and (B) LTL in animals ≤7.5 vs >7.5 years. Boxes show median and interquartile range, white circles show means, individual points show distribution. Significance asterisks (*** $p < 0.001$) and effect sizes shown.

3.4. Breed Differences

Significant breed differences were observed for multiple parameters (Kruskal-Wallis tests; Table 2). STL varied significantly among breeds ($H = 7.56$, $p = 0.023$), with Axis axis showing the highest values. Semen volume differed most markedly ($H = 23.1$, $p < 0.001$), with Rusa unicolor producing approximately twice the volume of Axis axis. Post-hoc pairwise comparisons revealed significant differences between all breed pairs for semen volume ($p < 0.01$) and between Axis axis and Rusa unicolor for STL ($p = 0.017$).

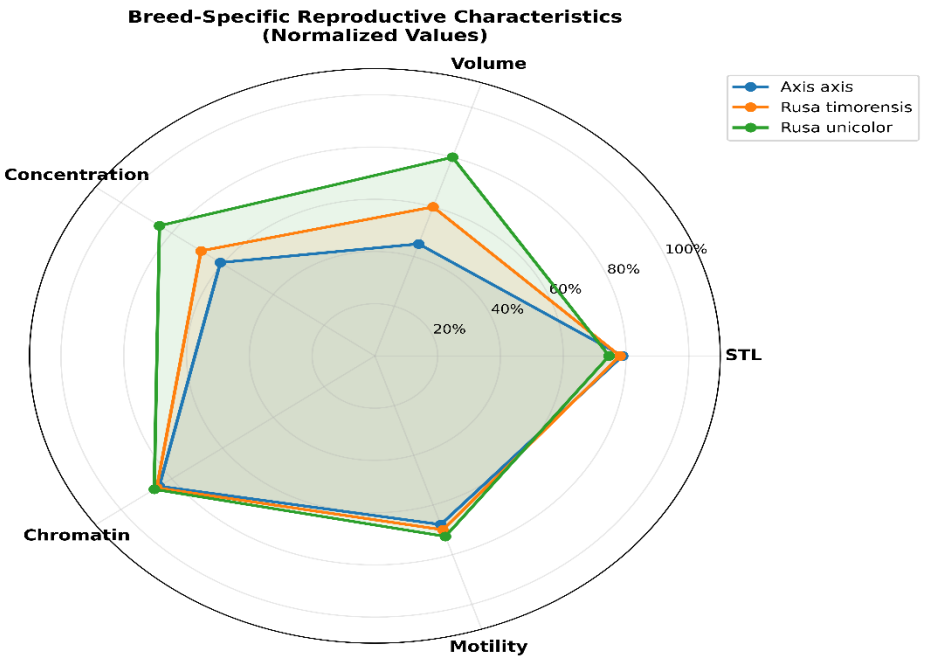

FIGURE 3: Radar chart comparing five reproductive parameters across three deer breeds. Each axis represents a normalized parameter (0-100%). Shows breed-specific patterns: *Axis axis* excels in STL but has lower volume, while *Rusa unicolor* shows opposite pattern.

Table 2. Breed comparison statistics (Kruskal-Wallis tests)

Parameter	H statistic	p-value	Post-hoc differences
STL	7.56	0.023	Axis axis> Rusa unicolor*
LTL	4.67	0.097	NS
Semen volume	23.1	<0.001	All pairwise p < 0.01
Sperm concentration	14.3	<0.001	Rusa unicolor > Axis axis*
Chromatin condensation	2.89	0.236	NS
Total motility	4.02	0.134	NS

p < 0.05; NS = not significant

3.5. Multivariate Analysis

Multivariate linear regression identified STL as the strongest predictor of sperm concentration (β = 0.58, p < 0.001), accounting for 92% of variance in a model including age, LTL, and oxidative

markers. For chromatin condensation, STL was again the primary predictor ($\beta = 0.67$, $p < 0.001$), explaining 98% of variance. Age contributed significantly to both models ($\beta = -0.31$ for concentration, $\beta = -0.25$ for chromatin; $p < 0.01$ for both), while oxidative markers showed no predictive value ($p > 0.05$).

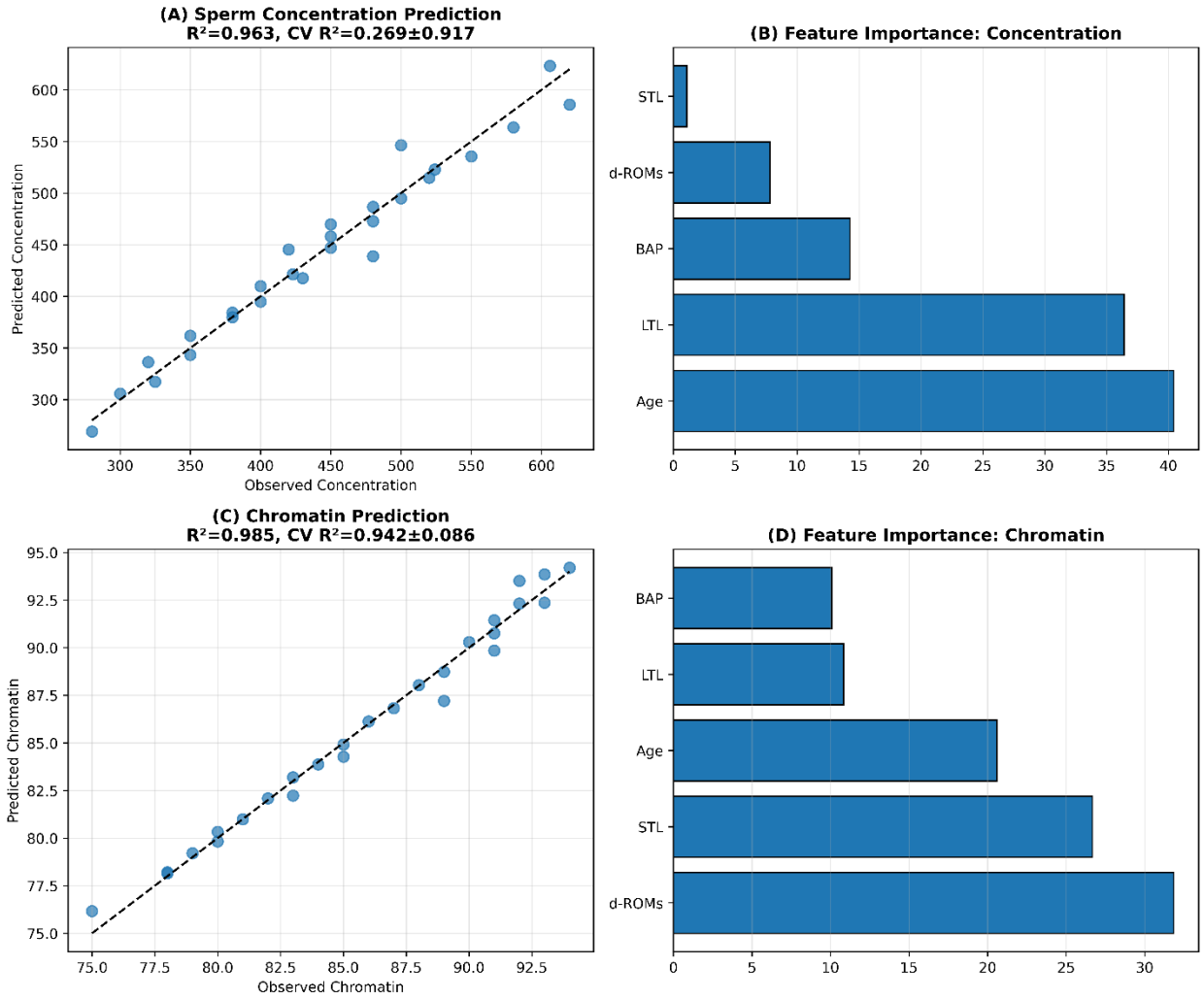

FIGURE 4: Four-panel figure showing regression model performance: (A) Observed vs predicted sperm concentration, (B) Feature importance for concentration prediction, (C) Observed vs predicted chromatin condensation, (D) Feature importance for chromatin prediction. STL consistently emerges as most important predictor.

3.6. Principal Component Analysis

PCA revealed distinct multivariate patterns (Fig. 3). The first three principal components explained 92.3% of total variance (PC1: 68.2%, PC2: 15.8%, PC3: 8.3%). PC1 was strongly loaded by STL (0.41), semen volume (0.39), and chromatin condensation (0.38), representing a "reproductive quality" axis. PC2 was loaded primarily by oxidative markers, and PC3 by breed-specific characteristics. Breed separation was evident along PC1, with Rusa unicolor clustering at higher reproductive quality values.

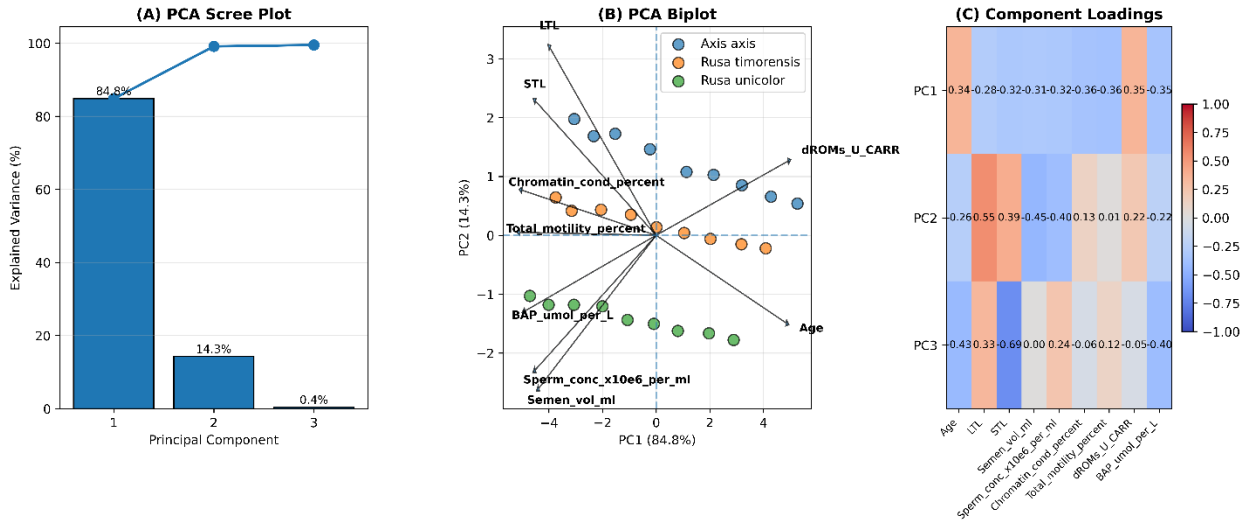

FIGURE 5: Three-panel figure: (A) Scree plot showing explained variance by principal components, (B) Biplot showing individual samples and variable loadings, (C) Heatmap of component loadings. PC1 represents "reproductive quality" axis, PC2 represents oxidative stress axis.

DISCUSSION

This study provides the first comprehensive analysis of telomere length dynamics in three deer species, revealing strong associations between STL, age, and semen quality parameters. The findings support STL as a sensitive biomarker of reproductive aging in cervids, with implications for breeding management and conservation.

The strong correlation between LTL and STL ($\rho = 0.94$) suggests coordinated telomere maintenance across somatic and germline compartments, contrasting with weaker correlations reported in humans [12] but aligning with findings in canines [9]. This high correlation supports the potential use of LTL as a less invasive proxy for STL in field conditions where semen collection is challenging. However, the earlier decline of STL (6 years) compared to LTL (7.5 years) indicates germline-specific vulnerability to aging processes, possibly due to higher oxidative stress exposure or replicative demands during spermatogenesis [13].

Breed-specific patterns revealed trade-offs between telomere maintenance and reproductive output. Axis axis, the smallest and shortest-lived species, exhibited the highest STL but lowest semen volume, suggesting investment in germline quality over quantity. Conversely, Rusa unicolor, larger and longer-lived, showed lower STL but higher semen volume, potentially reflecting different life history strategies. These patterns align with evolutionary theories predicting trade-offs between maintenance and reproduction [14].

The absence of correlation between TL and systemic oxidative markers contrasts with some human studies [15] but agrees with canine findings [9]. This may indicate: (1) local testicular oxidative stress is more relevant than systemic levels; (2) compensatory mechanisms protect telomeres from oxidative damage; or (3) our systemic markers inadequately captured oxidative stress relevant to telomere maintenance. Future studies should measure testicular-specific oxidative stress and antioxidant capacity.

STL's strong association with chromatin condensation ($\rho = 0.99$) suggests telomere integrity influences sperm DNA packaging, possibly through shelterin complex interactions or higher-order chromatin organization [16]. This relationship may explain observed associations between shorter telomeres and increased DNA fragmentation in other species [17].

Methodologically, our qPCR approach proved robust for deer samples, with amplification efficiencies and reproducibility comparable to established protocols [18]. The $\Delta\Delta$Ct method with GAPDH normalization provided reliable relative TL measurements, though absolute telomere length quantification would require additional validation.

Limitations

This study has several limitations. The cross-sectional design precludes causal inferences about telomere attrition over time. Small sample sizes per breed limit statistical power for detecting subtle breed differences. Systemic oxidative markers may not reflect testicular microenvironment conditions. Despite these limitations, the consistent, strong correlations suggest robust underlying relationships warranting further investigation.

Practical Implications

For deer breeding programs, STL assessment could enhance selection strategies by identifying animals with better reproductive longevity. Breed-specific TL baselines should be established to account for natural variation. The 6-year threshold for STL decline suggests optimal breeding age windows and potential interventions (e.g., antioxidant supplementation) before this critical point.

For conservation, TL monitoring could help assess population health and inbreeding effects, as telomere shortening accelerates under genetic stress [19]. Comparative studies across wild and captive populations could elucidate environmental influences on telomere dynamics.

CONCLUSION

This study establishes telomere length, particularly STL, as a valuable biomarker of reproductive aging in deer. Strong correlations between STL, age, and semen quality parameters, along with breed-specific patterns, provide insights into cervid reproductive biology. The findings support incorporating TL assessment into deer breeding programs to optimize reproductive management and conservation outcomes. Future longitudinal studies with larger samples and direct testicular oxidative stress measurements will further clarify telomere dynamics in cervids.

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