Theriogenology 254 (2026) 117827

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Original Research Article

Telomere lengths in blood and sperm as biomarkers of reproductive aging and semen quality in dogs

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ARTICLE INFO

Keywords:
Semen evaluation
Canine
Male fertility
Oxidative stress
Telomere shortening

ABSTRACT

To assess the potential utility of leukocytes and spermatozoa telomere length (LTL and STL) as reproductive biomarkers, this study measured both LTL and STL and investigated their possible correlations with oxidative status and semen quality parameters in healthy breeding male dogs. Twenty-two ejaculates and blood samples were collected from dogs of various breeds. Semen was evaluated for volume, concentration, sperm motility, and kinetic parameters using a Sperm Class Analyzer. LTL and STL were quantified using Quantitative PCR (qPCR) and expressed as the relative ratio of telomere repeat copy number (T) to a single-copy gene (S), T/S ratio. Serum oxidative stress markers were assessed using d-ROMs and Biological Antioxidant Potential (BAP) tests. Correlations between STL and LTL, as well as their association with age, semen parameters and oxidative stress levels, were evaluated using Spearman analysis. LTL and STL were 0.63 ± 0.25 and 0.85 ± 0.31 and showed a strong positive correlation ($P < 0.001$; $r_s = 0.70$). Both LTL and STL were also negatively correlated with age ($P < 0.05$ and $rs = -0.50$; $P < 0.001$ and $rs = -0.68$). The cut-off age for the difference in STL and LTL was identified at 6 and 7.5 years, respectively. STL was also positively correlated with semen volume ($P < 0.05$; $r_s = 0.63$) and concentration ($P < 0.05$; $r_s = 0.41$) and negatively correlated with semen chromatin decondensation ($P < 0.01$; $r_s = -0.71$); while the LTL showed a positive correlation with sperm concentration ($P < 0.05$; $r_s = 0.53$). No correlations with oxidative markers were found. These findings support the potential use of TL as a biomarker for reproductive aging and semen quality in dogs.

1. Introduction

As in other livestock species, hypo-infertility has recently been recognized as a cause of significant economic losses even in the dog breeding industry [1]. The progressive intensification of genetic selection in canine breeding, primarily focused on morphological or functional traits, has often overlooked other important characteristics, including fertility.

In some cases, selective breeding practices in purebred dogs have unintentionally affected reproductive performance, as evidenced by high rates of cesarean sections and reduced fertility, likely influenced by inbreeding and reduced genomic diversity [2,3]. A recent and striking example concerns cranial morphology in some canine breeds, which is incompatible with the transverse diameters of the maternal birth canal [4], making caesarean section up to 11 times more likely in brachycephalic breeds than in other breeds [3].

Recently, it has been demonstrated that the use of genomic data to calculate crossing coefficients is a more effective approach for assessing the loss of genetic diversity in canine populations and its detrimental impact on reproductive traits [2]. In this context, defining selection goals that integrate fertility-related traits and identifying reliable fertility biomarkers may support breeders in enhancing reproductive performance while preserving desirable breed characteristics.

Sperm telomere length (STL) has recently been recognized as a novel biomarker for the diagnosis of male infertility in humans [5]. A link between telomere length (TL) and male infertility has been suggested by the evidence showing a correlation between telomere shortening and sperm DNA damage [6]. Furthermore, a recent study reported that TL in both sperm and leukocytes is positively correlated with sperm concentration and total sperm count in healthy men [7]. Similarly, in the canine species, STL is gaining attention as a potential biomarker of male

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https://doi.org/10.1016/j.theriogenology.2026.117827
Received 25 November 2025; Received in revised form 3 January 2026; Accepted 9 January 2026
Available online 12 January 2026

Glossary

ALH	amplitude of lateral head
BAP	Biological Antioxidant Potential
BCF	beat cross frequency
Cq	Quantification cycle
d-ROMs	reactive oxygen metabolites
LIN	Linearity
LTL:	leukocyte telomere length
NTC	no-template control
POT1	Protection Of Telomeres 1
ROS	reactive oxygen species
RTL:	relative telomere length
SCA	Sperm Class Analyzer
STL:	sperm telomere length
STR	straightness
TIN2	TRF1-Interacting Nuclear Factor 2
TL:	telomere length
TRF2	Telomere Repeat Factor 2
VAP	average path velocity
VCL:	curvilinear velocity
VSL:	straight-line velocity
WOB	wobble

fertility. Recent studies in humans and dogs have shown that STL varies among individuals and is positively associated with key sperm quality parameters, such as motility and concentration [1,5]. Furthermore, a preliminary canine study, showed that sperm and leukocyte telomeres are readily measurable and display inter-individual variability, suggesting potential relevance for the assessment of male fertility [1].

From an etiopathogenetic perspective, telomere shortening is associated with telomeric friction, a process linked to oxidative stress, genotoxic agents, or genetic predisposition. Reactive oxygen species (ROS) oxidize the guanine-rich regions of telomeres, triggering a DNA damage response that leads to the loss of telomeric repeats [6,7] and contributes to biological ageing [8]. Additionally, genetic mutations affecting telomerase activity or telomere stability can cause a spectrum of clinical disorders known as telomeropathies, which particularly affect germ cells and are often characterized by shortened or dysfunctional telomeres [9].

The international literature extensively documents that sperm quality is strongly influenced by oxidative stress, a phenomenon that can impair mitochondrial function [10], induce structural abnormalities in the flagella [11], and cause DNA damage [2,7,12]. Moreover, high concentrations of ROS can induce DNA damage that leads to telomere shortening and consequent impairment of sperm function [1,5]. On the other hand, in dogs, longer telomeres and distinct expression profiles of shelterin complex genes, including TRF2 (Telomere Repeat Factor 2), POT1 (Protection Of Telomeres 1), and TIN2 (TRF1-Interacting Nuclear Factor 2), have been associated to increased sperm motility [13]. Furthermore, telomerase activity appears to be preserved in canine testicular tissues, playing a crucial role in maintaining telomere integrity within germ cells [14]. Consequently, integrating markers for oxidative stress and STL into reproductive evaluations could improve selection strategies in dog breeding, thereby optimizing both fertility outcomes and genetic diversity.

This study aimed to investigate the relationships among TL in spermatozoa and leucocytes, systemic oxidative status, and semen quality parameters in healthy breeding male dogs, to evaluate the potential utility of these biomarkers as complementary tools for reproductive assessment and selection in canine breeding programs.

2. Materials and methods

2.1. Experimental design and animals

A total of 22 dogs were enrolled in the study between March and December 2024. All animals came from the XXX breeder center located in the province of XXX, and were kept under identical nutritional, environmental, and social management conditions. The dogs ranged in age from 1 to 11 years (mean ± SD; 6.1 ± 3.5 years), and included various breeds and sizes, specifically Golden Retriever (n = 5), Poodle (n = 4), French Bulldog (n = 3), Jack Russell Terrier (n = 2), Shih Tzu (n = 2), Pug (n = 2), Doberman (n = 1), Cavalier King Charles Spaniel (n = 1), Little lion dog (n = 1), Neapolitan Mastiff (n = 1). Blood and semen collection were performed as part of routine reproductive evaluation in breeding males.

2.2. Inclusion and Exclusion criteria

Inclusion criteria.

- Good general health;
- Proven fertility (recognized stud dogs).

Exclusion criteria.

- Clinical illness;
- Reproductive disorders or failure to conceive in the past year;
- Ongoing treatment with drugs known to affect semen quality;
- Significant (>10 %) weight loss in the previous month;
- Behavioral disorders;
- Any condition interfering with protocol compliance.

For each enrolled subject, a medical record was created. All dogs underwent a general physical and reproductive examination, which included ultrasonography of the prostate, testes, and penis, as well as hematological and biochemical profiling. Blood and semen samples were collected on the same day.

2.3. Ethical approval

The protocol was approved by the Ethical Animal Care and Use Committee of the XXX (PG/2024/0023587 of February 27, 2024) and conducted in accordance with the ethical standards of the Declaration of Helsinki.

2.4. Biological sample collection

2.4.1. Blood sampling

Three mL of blood were drawn from the cephalic vein using a 21-gauge butterfly needle and transferred into EDTA and clot-activator tubes. EDTA samples were frozen at −20 °C for TL analysis. Serum was obtained by centrifugation of clot-activator tubes at 3000 rpm for 10 min and stored at −20 °C for oxidative stress analysis, which was performed within two months.

2.4.2. Semen collection and evaluation

Semen samples were collected after a five to seven-day period of sexual rest using digital manipulation in the presence of a bitch in estrus and an artificial vagina. Only the sperm-rich fraction was collected to avoid contamination from the pre-spermatic and prostatic fractions, known to negatively affect quality [15–17].

Undiluted ejaculates were stored in polypropylene tubes, shielded from light, and transported in a polystyrene foam box at ~20 °C, with

analysis performed within 3 h. Semen volume was recorded immediately using a graduated cylinders or pipettes; concentration, motility, and kinetics were assessed using the Sperm Class Analyzer (SCA; Microptic SL, Veterinary Edition, Barcelona, Spain) on a Nikon Eclipse E200 microscope.

Parameters included concentration (x 10^6 spermatozoa/ml), total motility (%), progressive motility (%), sperm subpopulations (rapid/medium progressive), average path velocity (VAP, μm/s), straight-line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), straightness (STR, %), linearity (LIN, %), wobble of the curvilinear trajectory (WOB, %), beat cross-frequency (BCF, beats/s), and amplitude of lateral head displacement (ALH, μm). The SCA system settings for canine semen classified as spermatozoa all particles sized between 10 and 80 μm^2 and defined progressively motile spermatozoa as those with a STR >75 %. The VCL threshold used to define slow-medium and rapid spermatozoa subpopulations were 50 and 100 μm/s, respectively; spermatozoa with VCL values below 10 μm/s were considered static, whereas those with VCL >150 μm/s and ALH >3.5 μm were classified as hyperactive. Image acquisition was performed at 60 frames per second with a minimum contrast of 35.

An aliquot of 0.5 ml of semen was stored at −80 °C for further evaluation. Specifically, after a maximum of 19 months the semen sample was thawed at room temperature, and sperm chromatin condensation was evaluated within 2 h using Diff-Quik® stain (Bio-Optica, Milan, Italy), as previously described by Mota and Ramalho-Santos (2006) [18] Briefly, 10 μL of semen were smeared onto glass slides using a coverslip and allowed to air-dry. The slides were then sequentially immersed for 10–20 s in each of the three Diff-Quik solutions (methanol, eosin and thiazine), rinsed with water to remove excess stain, and air-dried. Evaluation was performed under a light microscope (Leica DMLB; Wetzlar, Germany) at 10 × to 40 × magnification. As shown in Fig. 1, spermatozoa were classified into two categories: spermatozoa with light-stained heads/nuclei (normal chromatin condensation) and spermatozoa with dark-stained heads/nuclei (partial or complete chromatin decondensation). For each sample, 200 spermatozoa were counted to determine the percentage of cells with condensed chromatin.

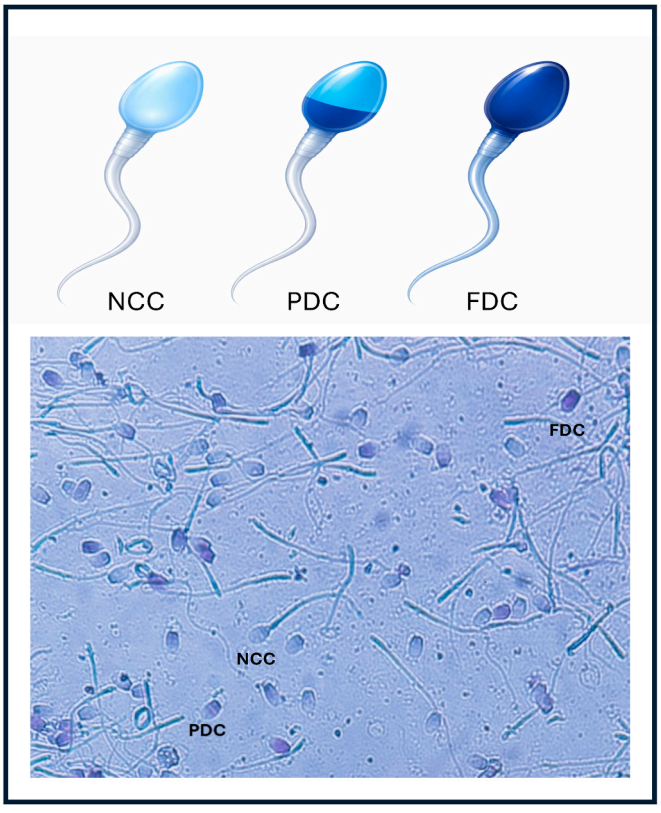

Fig. 1. Top panel: three categories of sperm head chromatin condensation: normally condensed (NCC); partially decondensed (PDC) and fully decondensed (FDC) sperm chromatin. Bottom panel: Photomicrograph of a dog sperm population (Diff-Quick stain, light microscope, 40 × magnifications) including the three categories of condensed/decondensed chromatin.

2.5. Oxidative stress evaluation

Serum levels of oxidative stress markers were measured using reactive oxygen metabolites (d-ROMs) and Biological Antioxidant Potential (BAP) tests (Diacron® kits, Grosseto, Italy), according to the manufacturer's protocol and validated methods [19]. Analyses were performed using the SAT450 analyzer (KPM Analytics, USA). The d-ROMs test measures hydroperoxide-derived radicals via a colorimetric reaction at 505 nm; with results expressed in Carratelli Units (U CARR); normal canine values ranged 50–90 U CARR. The BAP test assesses plasma antioxidant capacity via ferric ion reduction, with results expressed in mMol/L (normal canine range: 2200–2400 mMol/L) [20].

2.6. Blood and semen TL evaluation

Genomic DNA was isolated from sperm and blood samples using the Genejet DNA Purification Kit (ThermoFisher Scientific). DNA concentration and purity were assessed for each sample using a NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific). Prior to quantitative polymerase chain reaction (qPCR) analysis, all genomic DNA samples were diluted to a final concentration of 10 ng/μL. DNA quality met the following criteria: yield >30 ng/μL, 260/280 ratio >1.7, and 260/230 ratio >1.8.

All qPCR analysis were performed in accordance with established guidelines and regulations for qPCR experiments [21] and for TL measurement using qPCR approaches [22]. Relative telomere length (RTL) was measured using a 96-well CFX RT-PCR System (Bio-Rad) with a total reaction volume of 10 μL, containing 30 ng of genomic DNA, iTaq Universal SYBR Green Supermix (Bio-Rad), and forward and reverse primers for telomere repeats (T) [23] and GAPDH used as a single copy gene (SCG; NCBI Reference Sequence: NC_051831.1).

Each sample was assayed in triplicate (intra-assay) across two separate runs (inter-assay), including a no-template control (NTC). A standard curve for each primer was used to assess amplification efficiency and linearity.

Primer specificity was validated by melt-curve analysis. Each sample was run in triplicate across three separate experiments. Samples with coefficient of variation >5 % (n = 4) were excluded and reanalyzed. T/S ratios were calculated using the ΔΔCt method [24].

2.7. Statistical analysis

Data normality was assessed via the Shapiro-Wilk test. Due to non-normal distribution and small sample size, non-parametric tests were used. Correlations between STL and LTL, as well as between these variables and age, sperm quality parameters, and oxidative stress, were assessed using Spearman's correlation analysis. Spearman's correlation coefficient was interpreted as follow: complete (rs = 1), strong (0.70–0.99), moderate (0.50–0.69), and weak (0.01–0.49) [25]. Wilcoxon tests were used to compare age-based groups for leukocyte and sperm T/S ratios. Statistical analysis was performed using SPSS v29.0 (IBM Corp., Armonk, NY, USA), with significance set at $P \leq 0.05$.

3. Results

3.1. Semen

The ejaculates collected were white and milky in appearance, with

no macroscopical abnormalities observed. The volume of sperm-rich fraction averaged 1.8 ± 1.5 mL (mean ± SD), and the sperm concentration was 164.9 ± 114.9 x 10^6 spermatozoa/mL. Motility and kinetic parameters are reported in Table 1. The percentage of sperm chromatin decondensation was 51.1 ± 30.0 %.

3.2. Leukocyte and spermatozoa TL

Quantitative PCR amplification efficiencies were 98.3 % for T and 98.6 % for the SCG. Quantification cycle (Cq) values showed high reproducibility, with a standard deviation below 0.5. Two independent standard curves were generated for the telomere and SCG sequences, with SYBR Green fluorescence signals acquired at 64 °C for both T and SCG during each qPCR cycle. RTL was calculated as the T/S ratio (telomere repeats to SCG copies) using the ΔΔCt with Pfaffl correction [26].

The mean ± SD leukocyte and sperm T/S ratios were 0.63 ± 0.25 and 0.85 ± 0.31, respectively. A strong positive correlation between leukocyte and sperm T/S ratios was observed ($P < 0.001$; $r_s = 0.70$), as shown in Fig. 2.

3.3. Correlations between TL and oxidative stress

Serum d-ROMs concentration was 66.3 ± 51.8 UCARR, while BAP levels were 1611.4 ± 769.9 μmol/L. No correlation was observed between STL or LTL with oxidative stress.

Both leukocyte and sperm T/S ratios were also negatively correlated with age ($P < 0.05$ and $r_s = -0.50$; $P < 0.001$ and $r_s = -0.68$, respectively), as shown in Fig. 3.

The cut-off age for the difference in sperm T/S ratios was identified at 6 years. Dogs aged ≤6 years had higher ($P < 0.05$) sperm T/S ratios (1.01 ± 0.30) compared to dogs older than 6 years (0.68 ± 0.20). Meanwhile, a cut-off of 7.5 years was necessary to detect a difference in leukocyte T/S ratios. Indeed, dogs older than 7.5 years exhibited a lower leukocyte T/S ratio (0.51 ± 0.21) compared to those aged ≤7.5 years (0.72 ± 0.24). The leukocyte and semen T/S ratio distribution for age are shown in Fig. 3.

3.4. Correlations between TL and semen parameters

An overview of all correlations identified is presented in Fig. 4. Sperm T/S ratio was positively correlated with semen volume ($P < 0.05$; $r_s = 0.63$) and concentration ($P < 0.05$; $r_s = 0.41$), and negative correlated with sperm chromatin decondensation ($P < 0.01$; $r_s = -0.71$); while the leukocyte T/S ratio showed a positive correlation with sperm concentration ($P < 0.05$; $r_s = 0.53$). No correlations were observed between sperm chromatin decondensation and systemic oxidative stress markers, nor were there correlations between other semen parameters and oxidative markers were found.

Table 1
Mean and standard deviation (SD) of motilities and kinetic parameters of canine ejaculates used in this study.

Parameters	Value (mean ± SD)
Total Motility (%)	65.8 ± 28.5
Progressive Motility (%)	25.8 ± 16.5
Rapid (%)	2.6 ± 3.9
Medium (%)	28.2 ± 18.0
Slow (%)	35.0 ± 17.0
VCL (μm/s)	49.5 ± 19.1
VSL (μm/s)	36.1 ± 16.6
VAP (μm/s)	41.2 ± 18.5
LIN (%)	60.3 ± 20.6
STR (%)	70.0 ± 20.8
WOB	73.1 ± 21.5
BCF (beats/s)	6.4 ± 2.2
ALH (beats/s)	1.5 ± 0.5

Abbreviations: VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, Linearity; STR, straightness; WOB, wobble; BCF, beat cross frequency; ALH, amplitude of lateral head.

4. Discussion

In humans, TL has been widely studied as an indicator of cellular aging and reproductive health [27]. Over the past decades, research has focused on TL in peripheral blood leukocytes and reproductive cells, exploring its potential as a non-invasive biomarker for aging and fertility [27]. This study investigated the potential use of STL and LTL as supplementary biomarkers for reproductive evaluation in canine breeding programs. For the first time in dogs, a strong positive correlation was demonstrated between LTL and STL, along with a clear age-related decline in both cell types. Additionally, TL was positively correlated with sperm concentration; however, no correlations were found between TL and sperm motility, kinetic parameters, or oxidative stress markers.

A key finding of the present study in dogs was the strong correlation between leukocytes and spermatozoa TL, suggesting coordinated telomere dynamics across somatic and germinal compartments. This is relevant from a practical standpoint, as blood sampling is less invasive and more feasible in clinical or breeding settings where semen collection is not always possible. By contrast, in humans, correlations between LTL and STL are generally weak to moderate, likely reflecting tissue-specific regulatory mechanisms and differing telomere attrition rates among cell types [28,29]. Nevertheless, several studies have reported significant inter-individual correlations between LTL and STL, supporting the hypothesis of a shared genetic determination of baseline telomere length established early in life [30]. Moreover, domestic dogs share several aspects of telomere biology with humans, including comparable TL, similar attrition rates, and low or absent telomerase activity in normal somatic tissues. Nasir et al. [14] further showed that the distribution of telomerase activity in canine tissues closely resembles that observed in humans, with high activity in germinal and tumor tissues and minimal activity in normal somatic tissues. These features support the use of the dog as a valuable alternative model for studying telomere dynamics and age-related disorders, particularly given the limitations of classical murine models [31].

In the present study, both LTL and STL were negatively correlated with age, with a more pronounced and earlier decline observed in spermatozoa. Specifically, STL reduction was evident in dogs older than 6 years, whereas LTL shortening became significant only beyond 7.5 years of age. This temporal divergence suggests that the canine germinal compartment may be more sensitive to aging-related processes than somatic tissues, possibly due to increased cellular turnover or cumulative environmental stressors [32]. In somatic cells, telomeres shorten with each cell division as a consequence of the end-replication problem and limited telomerase activity, contributing to cellular senescence and aging [33]. Although germ cells exhibit higher telomerase activity, TL may nevertheless decline with age depending on environmental and individual factors [34].

These findings differ from observations in rodents, in which aging has been associated to a clear reduction in LTL, whereas STL significantly increased [35]. Furthermore, some human studies have reported stable or even increasing STL with age [30]. Such increases in human STL have been attributed to sustained telomerase activity during spermatogenesis and selective mechanisms favoring spermatozoa with longer telomeres across the reproductive lifespan [30,36]. These contrasting data highlight species-specific differences in germline telomere regulation [37].

Germline telomere regulation is highly species-specific and reflects evolutionary trade-offs among fertility, longevity, and cancer risk. Short-lived species tipically maintain long germline telomeres with relatively permissive telomerase activity, whereas long-lived species restrict telomerase more tightly, resulting in shorter but stable telomere

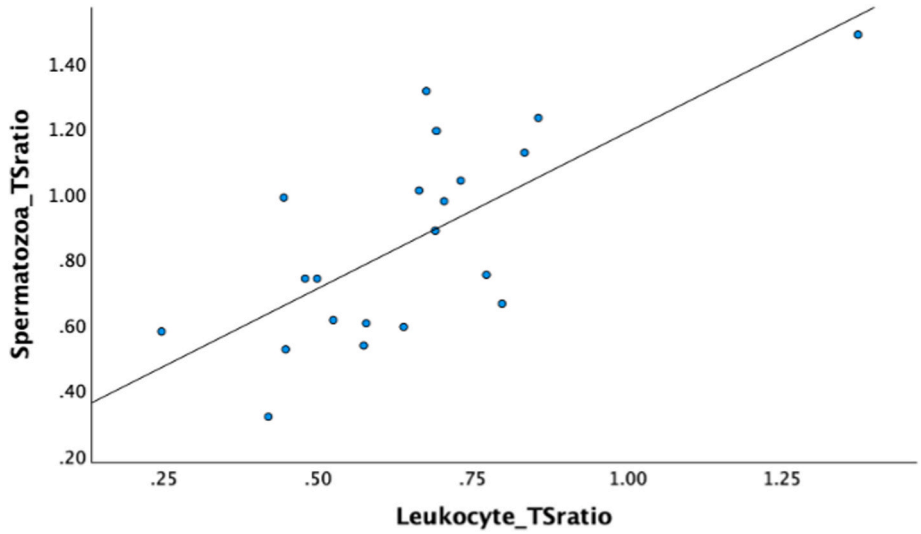

Fig. 2. Scatterplot illustrating a strong positive correlation between leukocyte and spermatozoa T/S ratios.

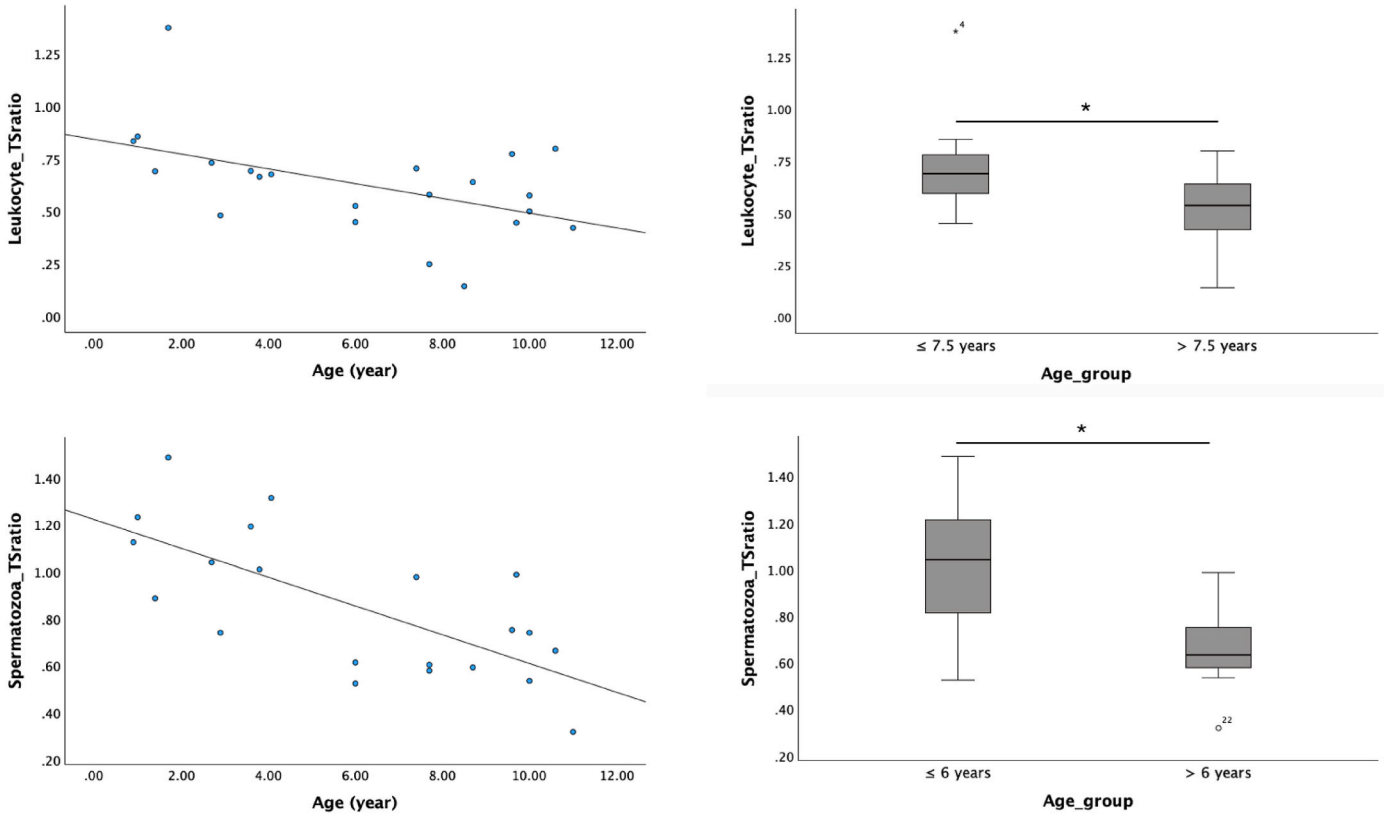

Fig. 3. Left panel: Scatterplots illustrating the negative correlation between leukocyte and spermatozoa T/S ratios and age. Right panel: Boxplots showing the distribution of spermatozoa T/S ratios in dogs aged ≤6 and > 6 years, and leukocyte T/S ratios in dogs aged ≤7.5 and > 7.5 years. Asterisks indicate between groups differences at $P \leq 0.05$.

length set points. Alternative strategies, such as retrotransposon-based telomere maintenance, further highlight evolutionary divergence [38–40].

In dogs, germline telomere length and its regulation are thought to influence aging and breed-specific lifespan. Indirect evidence suggesting that inherited telomere length set points and telomerase regulation in germ cells contribute to the accelerated aging observed in large breeds [32]. Our results show that the T/S ratio declines earlier in spermatozoa (6 years) than in leukocytes (7.5 years), highlighting fundamental differences in telomere maintenance and aging dynamics between germline and somatic tissues. In young dogs, active telomerase in spermatogonial stem cells maintains long telomeres in spermatozoa, supporting genomic integrity and fertility [38]. With age, germline telomerase activity may decline or become more tightly regulated, limiting telomere restoration during spermatogenesis and leading to earlier telomere attrition in sperm compared with leukocytes [32,41]. Spermatozoa are also highly susceptible to oxidative stress due to limited cytoplasmic antioxidant defenses and high mitochondrial

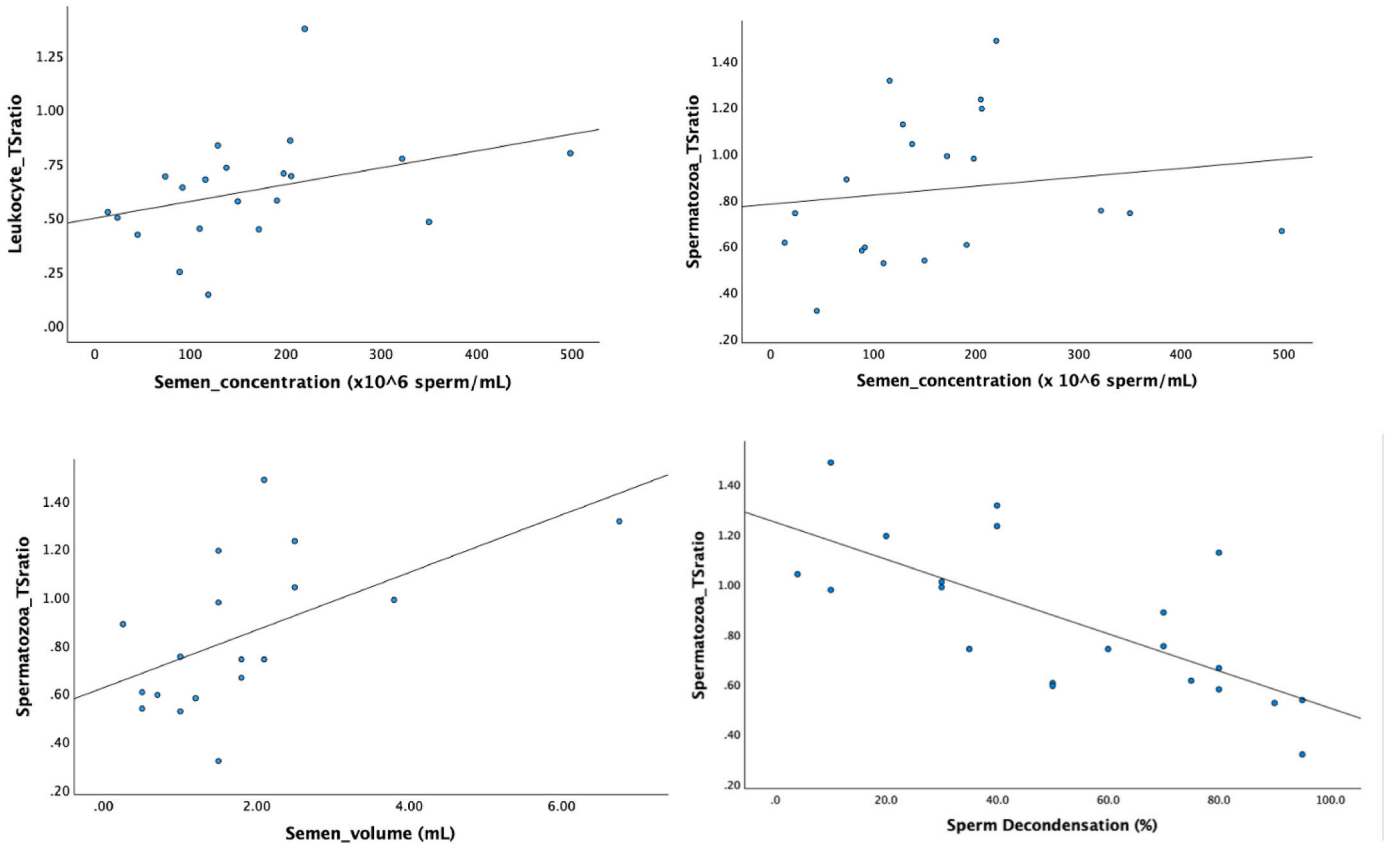

Fig. 4. Summary of scatterplots illustrating a Spearman correlation between leukocyte or spermatozoa T/S ratios and semen parameters (semen volume, concentration and sperm chromatin decondensation).

activity, which can accelerate telomere shortening independently of cell division [42]. In contrast, leukocytes possess more efficient DNA repair and antioxidant systems, resulting in slower telomere attrition and a delayed decline in the T/S ratio. Age-related changes in spermatogonial stem cell dynamics may further contribute, as clonal selection can favor spermatogonia with shorter telomeres but higher proliferative capacity, increasing heterogeneity and reducing average STL [32]. Continuous but slower leukocyte replication explains the delayed reduction in T/S ratio. Species- and breed-specific life-history traits may further modulate these patterns. In large, short-lived breeds, evolutionary pressures favor rapid growth and early reproduction over long-term germline maintenance, potentially contributing to the earlier STL decline [32,38]. The convergence of sperm and leukocyte T/S ratios in older dogs may reflect distinct aging trajectories, with leukocyte telomere attrition slowing at later ages while germline telomeres remain exposed to replicative and oxidative stress. Together, these mechanisms support the interpretation that canine germline telomere regulation is progressively compromised with age, potentially contributing to reproductive aging and reduced offspring telomere length in older males [41,42].

From a reproductive perspective, STL was positively correlated with ejaculate volume and sperm concentration and negatively correlated with chromatin decondensation. LTL also showed a positive association with sperm concentration, supporting a link between systemic telomere dynamics and spermatogenesis. However, no correlations were observed between TL and sperm motility or kinetic parameters. In humans, shorter STL has been associated with reduced sperm motility and infertility [43]. Similarly, Hassanpour et al. [1] reported altered expression of shelterin complex genes (TRF2, TIN2, POT1) in spermatozoa with poor motility, suggesting that telomere structure influences chromatin integrity and sperm function [44]. In rodents, longer TL has been linked to better sperm quality [45]. In cattle, Iannuzzi et al. [46] showed that bulls with longer TL exhibited enhanced motility, viability, membrane integrity, and fewer morphological anomalies, supporting TL as a quality marker. Overall, our results indicate an association between STL semen volume, sperm concentration and chromatin decondensation, although additional studies are required to clarify causality and to determine whether telomere erosion directly contributes to fertility impairment. Despite oxidative stress being implicated in aging and telomere attrition, our findings showed no correlation between systemic oxidative markers and TL in spermatozoa or leukocytes. This contrasts with studies in humans and rodents demonstrating that oxidative stress accelerates TL shortening [47–49]. In dogs, Lorke et al. [50] demonstrated that a diet enriched with antioxidants, mitochondrial cofactors, and omega-3 fatty acids may help preserve TL, suggesting that oxidative balance can influence telomere maintenance. However, systemic biomarkers may not accurately reflect local oxidative conditions affecting telomeres, and inter-individual variability, including age and breed, may have masked potential associations. Moreover, moderate oxidative stress may, in some cases, activate compensatory mechanisms that maintain or even elongate telomeres [51].

The first limitation of this study is that oxidative stress was assessed only in peripheral blood, without direct sperm-specific measurements, which limits the ability to draw conclusions regarding local reproductive oxidative stress within the reproductive tract [52]. However, a **chromatin condensation** test has been performed. Oxidative stress has been implicated in defects of chromatin condensation in both sperm and somatic cells, affecting sperm chromatin packaging by disrupting the non-random organization of mammalian chromosomes within the sperm nucleus [53].Therefore, the chromatin condensation test could be considered an indirect marker of sperm oxidative stress [54]. A second limitation is that the study population included multiple breeds, introducing potential breed-related heterogeneity in telomere length (TL),

which could not be statistically analyzed due to the low number of dogs per breed. To date, no studies have investigated the variability of STL among different canine breeds, although a recent study demonstrated that variability in TL in peripheral blood mononuclear cells is a strong predictor of average life span across 15 different dog breeds [33]. Nevertheless, expanding the dataset and conducting targeted studies on specific canine breeds and sizes represents an important objective for future research. Additional limitations include the experimental design, which, being cross-sectional, prevents causal inferences regarding telomere attrition and may increase variability in semen and telomere parameters, as reflected by the large standard deviations. Future longitudinal studies on larger, breed- and size-specific cohorts are needed to validate these findings and clarify the dynamics of LTL and STL in dogs.

In conclusion, this study provides novel insights into canine telomere biology, demonstrating a clear association between TL, age, and sperm concentration. The strong correlation between LTL and STL, along with their age-dependent decline, supports the use of TL as a marker of reproductive aging. Furthermore, the positive associations of STL with ejaculate volume, sperm concentration, and chromatin integrity reinforce its potential as a novel reproductive biomarker. Unlike in humans, where STL may be maintained or even increase with age, the canine germline undergoes age-related telomere shortening, which may contribute to declining reproductive performance. However, the association with systemic oxidative markers was weak, and further studies are needed to elucidate the relationship between STL and local oxidative stress. Overall, these findings highlight the value of the dog as a translational model for telomere-related fertility research and provide a rationale for incorporating TL, particularly STL, into assessments of male reproductive health in clinical and breeding contexts.

CRediT authorship contribution statement

Chiara Del Prete: Writing – original draft, Methodology, Investigation. **Alessandra Iannuzzi:** Writing – review & editing, Methodology, Conceptualization. **Valentina Longobardi:** Writing – review & editing, Investigation. **Maria Pia Pasolini:** Investigation, Writing – review & editing. **Ramona Pistucci:** Investigation. **Alfonso Calabria:** Investigation. **Bianca Gasparrini:** Supervision. **Natascia Cocchia:** Writing – original draft, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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