Theriogenology 189 (2022) 290–300

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Telomere length in bovine sperm is related to the production of reactive oxygen species, but not to reproductive performance

Jordi Ribas-Maynou [a, b, *], Marc Llavanera [a, b], Yentel Mateo-Otero [a, b], Nicolas Ruiz [a, b], Rodrigo Muiño [c], Sergi Bonet [a, b], Marc Yeste [a, b]

[a] Biotechnology of Animal and Human Reproduction (TechnoSperm), Institute of Food and Agricultural Technology, University of Girona, ES-17003, Girona, Spain
[b] Unit of Cell Biology, Department of Biology, Faculty of Sciences, University of Girona, ES-17003, Girona, Spain
[c] Department of Animal Pathology, Faculty of Veterinary Medicine, University of Santiago de Compostela, ES-15705, Lugo, Spain

ARTICLE INFO

Article history:
Received 16 February 2022
Received in revised form
20 April 2022
Accepted 22 June 2022
Available online 5 July 2022

Keywords:
Bull
Telomere length
Fertility
Sperm quality
ROS

ABSTRACT

Over the last decades, selection in cattle has mainly been based on milk production rather than on reproductive efficiency. While, when applied, focus on reproduction has involved females, attention has barely been paid to males and, if so, it has only looked at classical sperm quality parameters. In effect, variables such as telomere length have been missed, despite the fact that longer telomeres have been suggested to be linked to male fertility in humans. For this reason, the present study aimed to determine the length of telomeres in bovine sperm and their relationship with a) sperm quality evaluated through the conventional spermiogram and flow cytometry, and b) bull reproductive performance. For this purpose, 29 bulls were involved in this study. Sperm telomere length was evaluated through quantitative Fluorescent In Situ Hybridization (qFISH), and sperm quality was determined at 0 h and 4 h post-thaw. Bull fertility was assessed as non-return to estrus rates after 90 days of artificial insemination. Although the mean telomere length in bovine sperm was 12.06 ± 2.75 kb, the intra-individual variability in length led us to observe three different groups of telomeres in each sperm cell: short telomeres (7.14% ± 5.79% of telomeres; 8.29 ± 2.34 kb), medium telomeres (31.03% ± 12.92% of telomeres; 16.00 ± 2.72 kb) and long telomeres (61.93% ± 18.11% of telomeres; 30.13 ± 11.35 kb). Moreover, whereas reactive oxygen species (ROS) were found to be correlated to sperm telomere length (Rs = −0.492; P= 0.007), no correlation with other sperm quality parameters was found ($P > 0.05$). Reproductive performance after artificial insemination was not seen to be correlated to sperm telomere length (Rs = 0.123; P= 0.520). In conclusion, this study determined, for the first time, the mean telomere length in bovine sperm and also reported that there is a high variability within each sperm cell. Yet, while telomere length was found to be correlated to ROS generation, it was not related to bull reproductive performance.

1. Introduction

A strong selective pressure has been applied during the last decades in species of commercial interest to obtain better phenotypic, genetic and productivity traits, and to keep the best animals to maximize industry revenues. Specifically, in the dairy sector, animal selection based on milk yield traits has led to significant improvements in the efficiency of dairy cows [1]. Different reports, however, have suggested that milk production could be negatively correlated to cow fertility; hence, the selective pressure could have led to a reduction in reproductive performance [1–4]. In this context, implementing new analyses to validate animal selection criteria is much relevant, as many cofounding factors prevent conclusive outcomes, and the inclusion of genetic and genomic traits may sometimes lead to inaccurate or biased estimations [5–7].

Unlike selection based on female traits, improvements in bull performance have been largely ignored for decades; yet, male reproductive traits have not been specifically defined for selection by breeding associations [8]. With regard to this, however, previous research reported that artificial insemination using semen from

* Corresponding author. Biotechnology of Animal and Human Reproduction (TechnoSperm), Faculty of Sciences, University of Girona, C/ Maria Aurèlia Campany, 69; ES-17003, Girona, Spain.
E-mail address: jordi.ribasmaynou@udg.edu (J. Ribas-Maynou).

https://doi.org/10.1016/j.theriogenology.2022.06.025

subfertile bulls leads to low fertility rates, which again supports that setting diagnostic tests to predict male fertilizing ability in this species is imperative [9]. Whilst it is obvious that the assessment of bull fertility can be conducted through monitoring their reproductive performance in a large fertility-proven cow population, neither is this method efficient nor does it allow the analysis of a high number of animals. Hence, the lack of a proper technique evaluating bull fertility leads to the use of non-optimal sires, which, in turn, results in lower non-return rates to estrus after servicing cows. Reproductive performance is known to rely upon sperm quality and, at present, the conventional spermiogram in commercial samples includes sperm morphology, concentration, motility and viability, among other variables [8]. These determinations have much benefited from the introduction of computer-assisted sperm analysis (CASA) and flow cytometry tests, which have standardized the results and reduced the high variability associated to sperm evaluation [10–12]. While these improvements have certainly increased the reliability of sperm quality analysis, there is still a room of improvement regarding the prediction of bull fertility because other functional parameters are missed in the conventional assessment of semen, yet they are of interest for the dairy cattle industry [13]. Related to this, genetic aspects such as chromatin integrity or protamine deficiency have emerged as potential biomarkers for sperm fertilizing capacity [14–17]. Although telomere length has been assessed as a fertility biomarker in human sperm [18,19], only one study, suggesting an association with motility, viability and morphology, was conducted in bovine [20].

Telomeres are hexanucleotide-repeat sequences associated to protein complexes that not only have implications for chromosome end protection, but are also involved in spermatogenesis, as they participate in chromosomal alignment, pairing and synapsis [21,22]. Differences in telomere length have been found between species, individuals, and even cell types [23]; thus, telomere length in sperm should be measured when analyzing its reproductive effects in the male counterpart [22]. While the relationship between telomere length and sperm quality has been tested in humans, the results are inconsistent because some studies showed a correlation between these parameters [18,24–26], but others failed to observe any relationship [19,27,28]. This is also the case of fertility, as whereas some authors reported a relationship between sperm telomere length and fertility outcomes [18,25,27–29], others observed no differences between fertile and infertile individuals [19,30,31].

Against this background, the present work aimed to measure telomere length in bovine sperm using quantitative Fluorescent In Situ Hybridization, as well as to address whether differences in this variable exist between chromosomes, and bulls. Furthermore, the study also sought to determine whether telomere length in bovine sperm is related to sperm quality and *in vivo* fertilizing capacity.

2. Materials and methods

2.1. Animals and ejaculates

Eighty-seven ejaculates from 29 sexually mature Holstein bulls (1.5–2 years old) were used for the present study. Animals were housed and collected at Cenero Artificial Insemination Centre in Gijón, Asturias (Spain), in compliance with all the European Union regulations for animal husbandry. Bulls were fed a standard diet adjusted to sires intended to semen production for artificial insemination.

For each animal, three ejaculates were collected through an artificial vagina (internal temperature: 45 °C) in five-week intervals throughout the year. Upon collection, all sperm samples were confirmed to fulfill the quality standards, and were then cryopreserved following the protocol described below. At post-thaw, an equal amount of each of the three independent ejaculates coming from the same bull was mixed to obtain a pool. These frozen-thawed sperm pools (29 biological replicates) were used for further experiments. As semen samples were commercially available for artificial insemination and authors did not conduct any intervention, no approval from an ethics committee was required.

2.2. Sperm cryopreservation

After collection and before cryopreservation, all ejaculates were confirmed to meet the standard quality thresholds for bull sperm quality (volume: between 2 and 8 mL; concentration $\geq 10^9$ sperm cells/mL; and total motility ≥85%). Sperm were cryopreserved following a standard protocol [32]. Briefly, ejaculates were diluted to 92×10^6 sperm/mL using a commercial freezing medium (Bioxcell; IMV Technologies, L'Aigle, France) at 22 °C. Thereafter, sperm samples were cooled to 4 °C (rate: 0.2 °C/min), equilibrated at this temperature for 3 h, and packed into 0.25-mL straws. Straws were frozen in a controlled-rate freezer (Digit-cool; IMV Technologies) and the freezing program consisted of the following curve: from 4 °C to −10 °C at −5 °C/min; from −10 °C to −100 °C at −40 °C/min, and from −100 °C to −140 °C at −20 °C/min. Finally, straws were plunged into liquid nitrogen and stored in a nitrogen tank until used.

Thawing was conducted through immersion in a 38 °C water bath for 20 s; as aforementioned, an equal amount of each of the three independent ejaculates coming from the same bull was mixed to obtain a pool. A total of 29 pooled ejaculates, each coming from a separate bull, were used.

2.3. Artificial insemination

Artificial insemination (AI) outcomes per bull were recorded as non-return rates to estrus after 90 days of servicing heifers and cows. These rates corresponded to the proportion of females that did not return to estrus after 90 days of artificial insemination. An average of 2,293 females per bull was inseminated (minimum of 277 and maximum of 15,231 females per bull).

2.4. Evaluation of telomere length through Fluorescent In Situ Hybridization (qFISH)

To evaluate the absolute telomere length, a quantitative Fluorescent In Situ Hybridization (qFISH) method was conducted, following the protocol previously set for pig sperm [33]. This technique is based on performing qFISH in sperm whose chromatin has been previously decondensed, and on the use of calibration fluorescent spheres to determine the absolute telomere length. The previous decondensing sperm chromatin step (formation of haloes) much increases the sensitivity of this analysis. Telomere length was measured upon thawing.

2.4.1. Decondensation of sperm chromatin

A previously optimized protocol to decondense bovine sperm chromatin was conducted to obtain fully decondensed haloes [34]. In brief, samples were diluted to a final concentration of 10^6 sperm/mL, and mixed 1:2 (v:v) with 1% low melting point agarose at 37 °C. Following this, 6.5 μL of the agarose-sperm mixture was placed onto an agarose pre-treated slide using circular coverslips (8 mm in diameter) and was allowed to jellify on a cold plate at 4 °C for 5 min. Thereafter, slides were incubated in three sequential lysis solutions at room temperature. First, lysis 1 (0.8 M DTT, 0.8 M Tris, 1% SDS, pH = 7.5) for 30 min; then, lysis 2 (0.4 M Tris, 0.4 M DTT, 2 M NaCl,

50 mM EDTA, 1% Tween20, pH = 7.5) for 30 min; and, finally, lysis 3 (0.4 M Tris, 0.4 M DTT, 2 M NaCl, 50 mM EDTA, 1% Tween20, 100 μg/mL proteinase K, pH = 7.5) for 3 h. Lastly, slides were washed in PBS for 5 min, and dehydrated in an ethanol series (70%, 90% and 100%, for 2 min each).

2.4.2. Analysis of telomere length through quantitative Fluorescent In Situ Hybridization (qFISH)

In order to conduct qFISH, slides with decondensed sperm were first fixed with 10% formaldehyde in PBS for 12 min, then washed in PBS for 5 min, dehydrated in an ethanol series (70%, 90% and 100%, for 2 min each), and finally dried horizontally. Following this, DNA was denatured through incubation in an alkaline solution containing 0.5 M NaOH for 4 min, and again dehydrated in an ethanol series and dried in horizontal position. Subsequently, the Peptide Nucleic Acid (PNA) probe (TTAGGGTTAGGGTT, Panagene; Korea) diluted in hybridization buffer (10 mM Na_2HPO_4, 10 mM NaCl, 20 mM Tris, 70% formamide, pH = 7.4) was denatured at 72 °C for 6 min, placed in the top of each sample and covered with a squared piece of parafilm. Hybridization was conducted in a dark damp container at room temperature for 1 h. Afterwards, parafilm was removed and slides were washed in PBS +0.1% Tween20 at room temperature for 2 min, then washed in PBS +0.1% Tween20 at 48 °C for 20 min, and finally in 2 × SSC +1% Tween20 at room temperature for 2 min. After ethanol dehydration and drying in horizontal position in a dark room, samples were mounted in antifading medium containing DAPI to counterstain nuclei (ThermoFisher Scientific, Whaltham, MA, USA), and stored at 4 °C. Imaging was performed on the following day.

2.4.3. Imaging and absolute Telomere length analysis

Telomere signals were visualized under a Zeiss epifluorescence microscope (Axio Imager.Z1, Carl Zeiss AG; Oberkochen, Germany) through the *filter set 40* (Carl Zeiss AG; Oberkochen, Germany). Images for at least 45 cells per sample were captured (average: 50 cells; minimum: 45 cells; maximum: 66 cells) at 1000 × magnification after 1 s of exposure, using an AxioCam (Carl Zeiss AG; Oberkochen, Germany) and the AxioVision software (AxioVs40 V 4.6.1.0, Carl Zeiss AG; Oberkochen, Germany). This exposure time was previously set to prevent fluorescence intensity saturation.

Fluorescence intensity for each telomere signal was analyzed using TFL-Telo v2 software (British Columbia Cancer Centre; Vancouver, Canada), obtaining the raw fluorescence intensity for each telomere, background intensity and the area covered by the signal. After subtracting the background and eliminating negative intensity values, the intensity/area (named I/A) was used as an indicator of telomere length.

According to our previous set up, the measurement of (I/A) of SPHERO calibration particles (Rainbow Calibration, 8 peaks, 3.0–3.4 μm (FITC); Spherotech, USA) with a known number of FITC molecules attached was used to determine telomere length. This procedure took into consideration that our PNA probe contained a single FITC molecule for the 14 base pairs, and allowed to establish the following equation between I/A and telomere length in base pairs:

$$\text{Telomere length (kb)} = 1.625 \times \text{Fluorescence intensity (I/A)} - 2.812$$

2.5. Evaluation of sperm motility

Motility was evaluated at 0 h and 4 h post-thawing using a computer-assisted sperm analysis system (CASA) integrated with the ISAS software (Integrated Sperm Analysis System V1.0; Proiser SL, Valencia, Spain), under a negative phase-contrast field microscope (Olympus BX41 with 10 × 0.30 PLAN objective; Olympus, Tokyo, Japan). First, three μL of each sperm sample at 38 °C was loaded into a 20-μm pre-warmed Leja chamber slide (Leja Products BV; Nieuw-Vennep, The Netherlands). Then, videos were taken at 30 frames per sec including a total of 1,000 sperm per replicate.

Two technical replicates were analyzed to obtain the following parameters: percentages of sperm with rapid, medium, and slow motility; curvilinear velocity (VCL; instantaneous sequential progression along the trajectory; μm/s); straight-line velocity (VSL; straight sperm trajectory per second; μm/s); average path velocity (VAP; mean sperm trajectory per second; μm/s); linearity coefficient (LIN = VSL/VCL x 100; %); straightness coefficient (STR = VSL/VAP x 100; %); wobble coefficient (WOB: VAP/VCL x 100; %); mean amplitude of lateral head displacement (ALH; mean amplitude of the lateral oscillatory movement of the sperm head around the mean trajectory; μm); and frequency of head displacement (BCF; the number of sperm head lateral oscillatory movements around the mean trajectory per unit of time; Hz).

For total motility, a sperm cell was considered motile when the average path velocity (VAP) was higher than 10 μm/s, and it was considered with progressive motility when its straightness index (STR) was higher than 70%.

2.6. Evaluation of sperm morphology

For the determination of sperm morphology, thawed samples at 38 °C were incubated in 2% formaldehyde in PBS for 5 min at room temperature. Next, 100 sperm per sample and replicate were classified as normal or abnormal sperm (differentiating abnormal head size, shape or acrosome abnormalities; folded, broken or coiled tails; proximal or distal droplets; and isolated heads). Two replicates were evaluated, and the mean proportions of morphologically normal sperm were calculated.

2.7. Flow cytometry

Sperm viability, intracellular levels of reactive oxygen species, membrane lipid disorder and protamine deficiency were analyzed at 0 h and 4 h post-thawing using a CytoFLEX flow cytometer (Beckman Coulter; Fullerton, CA, USA) equipped with red, blue and violet lasers (637 nm, 488 nm and 405 nm, respectively). Flow cytometry analysis was performed using CytExpert Software (Beckman Coulter; Fullerton, CA, USA). For all parameters, incubations with fluorochromes were conducted in samples adjusted at 1×10^6 sperm/mL in pre-warmed PBS, evaluating two replicates per sample and analyzing 10,000 events per replicate.

SYBR-14, YO-PRO-1 and H_2DCFDA were excited with the 488 nm laser and their fluorescence was detected by the FITC channel (525/40). HE was excited with the 488 nm laser and its fluorescence was detected through the PE channel (585/42). Merocyanine 540 (M540) was excited with the 488 nm laser and detected by the ECD channel (610/20). PI was excited with the 488 nm laser and its fluorescence was detected through the PC5.5 channel (690/50).

2.7.1. Sperm viability (SYBR-14/PI)

Sperm viability was assessed after staining with SYBR-14 (final concentration: 32 nmol/L) and propidium iodide (PI) (final concentration: 7.5 μmol/L) at 38 °C for 15 min in the dark. Percentages of viable sperm were determined as percentages of green-stained sperm (SYBR-14^+/PI^-).

2.7.2. Membrane lipid disorder analysis (merocyanine 540)

Membrane lipid disorder was evaluated through staining using Merocyanine 540 (M540), which intercalates in the outer

monolayer of plasma membrane when the packing order of phospholipids decreases. Samples were incubated with M540 (final concentration: 10 nmol/L) and YO-PRO-1 (final concentration: 31.25 nmol/L) at 38 °C for 15 min in the dark. Four subpopulations were identified: viable sperm with low membrane lipid disorder ($M540^-$/YO-PRO-1^-); non-viable sperm with low membrane lipid disorder ($M540^-$/YO-PRO-1^+); viable sperm with high membrane lipid disorder ($M540^+$/YO-PRO-1^-); and non-viable sperm with low membrane lipid disorder ($M540^+$/YO-PRO-1^+).

2.7.3. Intracellular levels of ROS (H_2DCFDA)

Intracellular ROS levels were detected through staining with H_2DCFDA, which is oxidized into DCF^+ in the presence of reactive oxygen species. Samples were incubated with H_2DCFDA (final concentration: 100 μmol/L) and PI (final concentration: 5.6 μmol/L) at 38 °C for 20 min. Four subpopulations were identified in dot-plots: viable sperm with low levels of ROS (DCF^-/PI^-); non-viable sperm with low levels of ROS (DCF^-/PI^+); viable sperm with high levels of ROS (DCF^+/PI^-); and non-viable sperm with high levels of ROS (DCF^+/PI^+).

2.7.4. Sperm intracellular levels of superoxides (HE)

Superoxide levels ($O_2^-\cdot$) were detected through staining with hydroethidine (HE), which is oxidized into ethidium (E^+) and other products in the presence of $O_2^-\cdot$. Samples were incubated with HE (final concentration: 5 μmol/L) and YO-PRO-1 (final concentration: 31.25 nmol/L) at 38 °C or 20 min. Fluorescence spill-over of HE into YO-PRO-1 was compensated (2.24%), and fluorescence spill-over of YO-PRO-1 into HE was also compensated (7.50%). Four subpopulations were identified: viable sperm with low levels of superoxides (E^-/YO-PRO-1^-); non-viable sperm with low levels of superoxides (E^-/YO-PRO-1^+); viable sperm with high levels of superoxides (E^+/YO-PRO-1^-); and non-viable sperm with high levels of superoxides (E^+/YO-PRO-1^+).

2.8. Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics 25.0 (IBM Corp., Armonk, NY, USA), and graphs were generated using GraphPad Prism v.8 (GraphPad Software, La Jolla, CA, USA). Previous to further statistical analyses, data were checked for normal distribution (Shapiro-Wilk test), and homoscedasticity (Levene test). Differences between 0 h and 4 h post-thawing were assessed through the Wilcoxon test. Correlations between telomere length and all the other parameters were assessed through the Spearman test.

In order to identify the presence of sperm subpopulations related to telomere length, a cluster analysis using the between-groups linkage method based on the Euclidean distance was conducted. The different telomere length subpopulations in each sample were expressed as proportions.

Based on fertility data (non-return rates at 90 days post-AI), bulls were split into three groups: low fertility (<25th percentile), medium fertility (25th to 50th percentile) and high fertility (>75th percentile). Comparisons involving telomere or fertility groups were conducted through a non-parametric one-way ANOVA (Kruskal-Wallis test), using the Mann-Whitney test for pair-wise comparisons. Due to the low sample size (n = 29), the level of significance to consider results statistically significant was set at $P \leq 0.01$.

3. Results

3.1. Telomere length in bovine sperm

First, sperm telomere length was determined in each of the 29 pools of samples through the qFISH method (Fig. 1); an average ± standard deviation (SD) of 23.3 ± 15.8 telomeres per cell was analyzed. Telomere length ranged between 8.31 kb and 17.46 kb, the mean was 12.06 ± 2.75 kb, and the 25th and 75th percentiles were 10.23 kb and 14.47 kb, respectively (Fig. 2A and Table 1A). Thanks to the use of qFISH, individual telomere signals could be assessed, showing a variable telomere length among different sperm cells from the same individual. The average dispersion (average standard deviation) of intra-individual telomere length was 7.11 kb, the range being between 4.28 kb and 10.23 kb (Supplementary Table 1).

The aforementioned variability also suggested the presence of different telomere lengths among the different chromosomes of the same sperm cell. A clustering analysis including all single telomere signals was thus run to classify the telomeres based on their length. Three clusters built on the basis of telomere length were identified: i) long telomeres (length: 30.12 ± 11.35 kb; minimum: 22.28 kb; maximum: 138.29 kb); ii) medium telomeres (length: 16.00 ± 2.72 kb; minimum: 12.20 kb; maximum: 22.27 kb); and iii) short telomeres (length: 8.29 ± 2.34 kb; minimum: 1.42 kb; maximum: 12.20 kb). The proportions of each of these clusters in every sperm sample were calculated, indicating the percentage of telomeres belonging to each cluster. The average incidences of each telomere group considering all samples were 7.14% ± 5.79% (incidence of long telomeres), 31.03% ± 12.92% (incidence of medium-size telomeres) and 61.93% ± 18.11% (incidence of short telomeres) (Fig. 2B and Supplementary Table 1).

3.2. Sperm quality

3.2.1. Sperm morphology, motility and kinematic parameters

For all biological replicates, sperm morphology was analyzed after thawing (Table 1A). Post-thaw sperm motility and kinematic parameters were analyzed at both 0 h post-thaw and after 4 h of incubation at 38 °C. A statistically significant reduction in most analyzed parameters was observed ($P < 0.01$), except for the percentages of sperm with medium and slow velocity (Table 1A).

3.2.2. Flow cytometry parameters: viability, membrane lipid disorder, intracellular superoxide and intracellular ROS

Table 1B shows sperm variables evaluated by flow cytometry (viability, membrane lipid disorder, ROS and superoxides) at both 0 h post-thaw and after 4 h of incubation at 38 °C. In most cases, a significant time-dependent deterioration was observed ($P < 0.001$), except for non-viable sperm with low lipid disorder, non-viable sperm with high ROS and non-viable sperm with low superoxides ($P > 0.01$)

3.3. Relationship between telomere length and sperm quality

In order to evaluate the relationship between sperm telomere length and sperm quality, the Spearman test was conducted using average telomere length, incidence of long and medium telomeres, and the aforementioned sperm quality parameters. No statistically significant correlation between mean telomere length and sperm morphology, motility, viability, membrane lipid disorder or the presence of high superoxide content was observed (Table 2). In contrast, a negative correlation was identified between both telomere length and the proportion of medium telomeres with the percentage of non-viable sperm with high ROS levels at 4 h post-thawing (Rs = −0.492; P= 0.007 and Rs = −0.555; P= 0.002) (Fig. 3).

3.4. Relationship between telomere length and AI outcomes

Non-return rates after 90 days of artificial insemination (NRR)

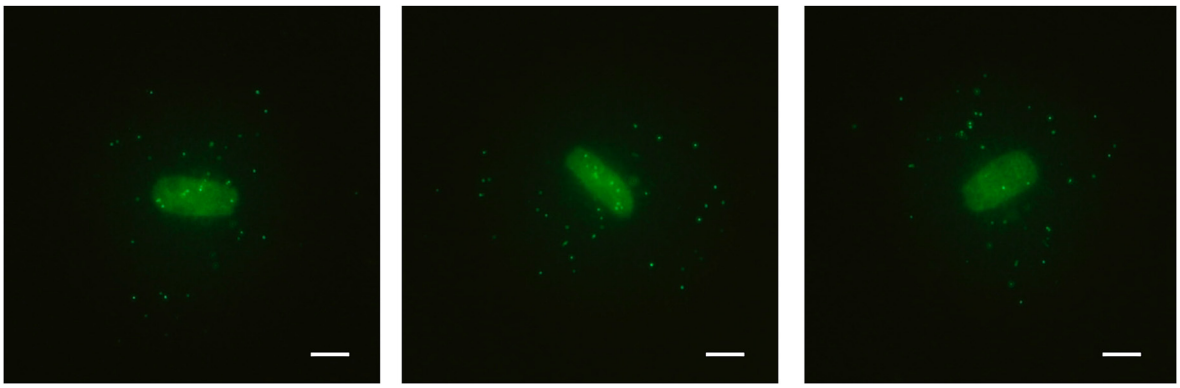

Fig. 1. Representative images of quantitative fluorescent in situ determination (qFISH) of telomere length in sperm haloes. Scale bar represents 5 μm.

were not found to be correlated to mean telomere length (Rs = 0.123; *P*=0.52) (Fig. 4A) or proportions of long (Rs = 0.197; *P*=0.306), medium (Rs = 0.126; *P*=0.514) or short telomeres (Rs = −0.156; *P*=0.419).

Another statistical approach consisted of classifying bulls into three groups of fertility based on their NRR. Animals were thus classified as with low (<25th percentile; NRRs ranging from 33.33% to 36.86%); medium (25th to 75th percentiles; NRRs ranging from 37.60% to 40.37%); or high fertility (>75th percentile; NRR ranging from 40.60% to 43.48%). Following this, the average telomere length and the incidence of long, medium and short telomeres in each sample were compared between the three groups of fertility. Mean telomere length was 12.37 ± 2.43 kb, 11.18 ± 2.57 kb and 13.81 ± 2.84 kb, for low, medium and high fertility groups, respectively (*P*=0.139). The incidence of long telomeres was 7.00% ± 5.73%, 5.44% ± 4.62% and 11.14% ± 7.03%, for low, medium and high fertility groups, respectively (*P*=0.168). The incidence of medium telomeres was 32.67 ± 11.15%, 27.13 ± 13.16% and 38.57 ± 11.5%, for low, medium and high fertility groups, respectively (*P*=0.161). The incidence of short telomeres was 60.33 ± 16.93%, 67.46 ± 17.04% and 50.43 ± 17.99%, for low, medium and high fertility groups, respectively (*P*=0.164) (Fig. 4B).

Sperm quality parameters between the three fertility groups were also compared, and results are shown in Table 3. Among all parameters, only the straightness coefficient was significantly different between low and medium fertility groups (*P*= 0.003).

4. Discussion

To the best of the authors' knowledge, this is the first study having determined telomere length in bovine sperm through quantitative Fluorescent In Situ Hybridization, using a cohort of 29 bulls. On the one hand, we observed that not only does sperm telomere length vary between animals, but also among sperm cells of the same ejaculate and even among chromosomes of the same sperm cell; thus, one may find telomeres with different length in a given spermatozoon. The assessment at single telomere level led us to identify three different clusters according to telomere length, which allowed establishing the relative incidence of each telomere length group in every sperm sample; remarkably, this could be used to test the relationship with the characteristics of each sample. On the other hand, while sperm telomere length was not found to be associated to conventional spermiogram variables, it was observed to be correlated to the proportions of sperm with high ROS. This suggests that high intracellular levels of ROS could lead to shorter sperm telomere length, even though whether high ROS levels during spermatogenesis could impair telomerase activity or these ROS could damage telomeres after spermiation is not yet clear. Finally, fertility data expressed as non-return to estrus rates after 90 days of AI were not found to be correlated to sperm telomere length.

Our study shows that telomere length in bovine sperm ranges between 8.31 kb and 17.46 kb, with an average of 12.06 kb. As far as

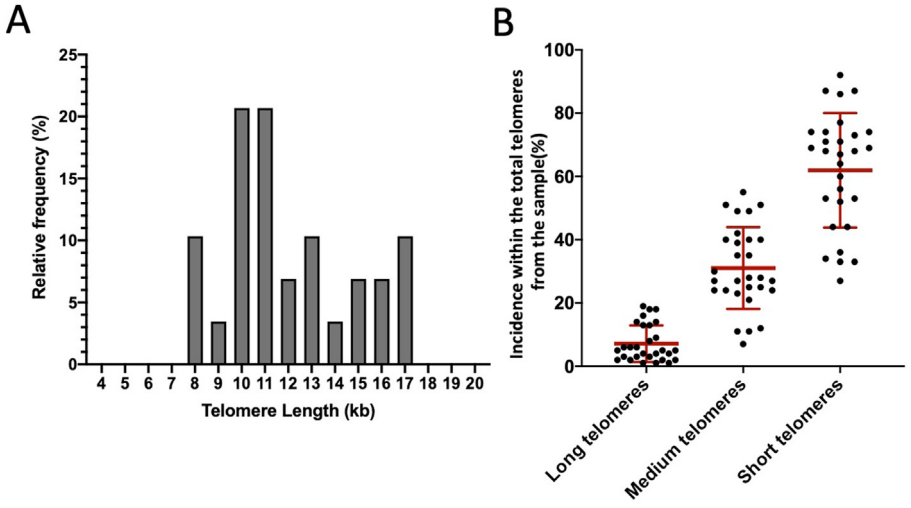

Fig. 2. Telomere length in bovine sperm. (A) Histogram representing the relative frequency of the observed telomere length in bovine sperm. (B) Incidence of each telomere cluster in each sperm sample.

Table 1
Descriptive variables analyzed throughout the study at 0 h and 4 h post-thawing. (A) Shows telomere length, morphology and kinematic parameters; (B) shows flow cytometry variables (viability, membrane lipid disorder and reactive oxygen species).

A									
	T = 0h				T = 4h				
	Mean	Standard deviation	25% percentile	75% percentile	Mean	Standard deviation	25% percentile	75% percentile	*P* value
Telomere Length (kb)	12.06	2.75	10.23	14.47					
% Long telomere length	7.14	5.79	2.50	13.00					
% Medium telomere length	31.03	12.92	24.00	40.00					
% Short telomere length	61.93	18.11	48.00	74.00					
Normal morphology (%)	89.13	3.36	86.40	91.75					
Abnormal morphology (%)	10.87	3.36	8.25	13.60					
Head abnormalities	0.80	0.78	0.00	1.44					
Cytoplasmic droplets	0.61	0.74	0.00	0.99					
Tail abnormalities	6.82	2.93	4.50	9.01					
Isolated heads	2.63	1.53	1.95	3.38					
Total Motility (%)	47.03	14.04	39.21	56.89	26.10	16.51	15.75	32.66	**<0.001** *
Progressive Motility (%)	27.19	9.62	19.70	32.88	13.17	9.28	7.15	19.78	**<0.001** *
Fast velocity (%)	30.10	11.74	21.66	36.44	12.82	10.63	4.51	19.14	**<0.001** *
Medium velocity (%)	9.00	2.72	7.30	11.15	8.44	3.96	6.36	10.82	0.456
Slow velocity (%)	8.58	8.01	3.04	11.51	4.84	5.23	2.06	4.09	0.013
VCL (μm/s)	80.39	17.35	68.26	91.84	52.61	15.00	43.71	64.74	**<0.001** *
VSL (μm/s)	42.55	13.29	31.40	48.92	22.68	10.83	14.05	30.16	**<0.001** *
VAP (μm/s)	48.90	13.46	37.89	55.08	28.82	10.72	20.96	37.24	**<0.001** *
LIN (%)	51.90	6.64	49.46	54.77	41.07	9.21	33.62	48.84	**<0.001** *
STR (%)	86.00	4.69	83.79	89.28	75.67	10.37	69.31	83.44	**<0.001** *
WOB (%)	60.12	5.18	57.79	62.59	53.67	5.47	49.58	58.56	**<0.001** *
ALH (μm)	3.07	0.51	2.69	3.36	2.19	0.53	1.94	2.49	**<0.001** *
BCF (Hz)	12.19	1.35	11.64	13.25	8.84	3.46	6.90	11.74	**<0.001** *
B									
Viability (%)	51.23	10.45	45.10	59.58	37.46	11.26	31.19	46.60	**<0.001** *
Membrane lipid disorder									
Live sperm with lipid disorder ($M540^+/YO\text{-}PRO\text{-}1^-$)	5.65	1.68	4.24	6.77	3.74	1.22	2.98	4.70	**<0.001** *
Live sperm without lipid disorder ($M540^-/YO\text{-}PRO\text{-}1^-$)	53.69	9.95	47.48	60.99	38.10	11.95	32.16	46.95	**<0.001** *
Dead sperm with lipid disorder ($M540^+/YO\text{-}PRO\text{-}1^+$)	40.57	10.05	33.59	45.79	58.12	12.57	48.48	64.08	**<0.001** *
Dead sperm without lipid disorder ($M540^-/YO\text{-}PRO\text{-}1^+$)	0.09	0.12	0.02	0.10	0.05	0.07	0.01	0.08	0.022
Intracellular ROS content									
Live sperm with high ROS ($H2DCFDA^+/PI^-$)	5.22	4.01	2.42	6.14	1.81	2.12	0.50	2.22	**<0.001** *
Live sperm with low ROS ($H2DCFDA^-/PI^-$)	39.96	9.37	34.17	45.21	30.41	12.28	22.34	40.88	**0.001** *
Dead sperm with high ROS ($H2DCFDA^+/PI^+$)	0.48	0.08	0.42	0.54	0.47	0.09	0.39	0.54	0.909
Dead sperm with low ROS ($H2DCFDA^-/PI^+$)	44.15	14.23	33.69	56.00	55.98	13.37	46.58	64.30	**<0.001** *
Intracellular superoxide content									
Live sperm with high superoxides ($HE^+/YO\text{-}PRO\text{-}1^-$)	3.96	2.77	2.52	4.09	0.84	0.61	0.34	1.29	**<0.001** *
Live sperm with low superoxides ($HE^-/YO\text{-}PRO\text{-}1^-$)	47.24	8.91	43.28	53.81	41.51	8.82	36.39	49.52	**0.004** *
Dead sperm with high superoxides ($HE^+/YO\text{-}PRO\text{-}1^+$)	43.63	9.60	35.69	48.67	54.93	9.41	47.29	60.22	**<0.001** *
Dead sperm with low superoxides ($HE^-/YO\text{-}PRO\text{-}1^+$)	5.17	3.98	1.99	7.89	2.72	0.98	2.02	3.03	0.017

Table 2
Correlation coefficients and P-values for total telomere length and proportions of medium telomeres with regard to: (A) morphology and motility, and (B) viability, membrane lipid disorder and reactive oxygen species.

A	Total telomere length				Proportion of medium telomeres			
	T = 0h		T = 4h		T = 0h		T = 4h	
	Rs	*P*-value	Rs	*P*-value	Rs	*P*-value	Rs	*P*-value
Normal morphology (%)	0.114	0.557			0,015	0,938		
Abnormal morphology (%)	−0.149	0.441			−0,052	0,787		
Head abnormalities	−0.133	0.493			−0,074	0,702		
Cytoplasmic droplets	0.145	0.453			0,200	0,300		
Tail abnormalities	−0.114	0.557			−0,019	0,924		
Isolated heads	−0.150	0.437			−0,153	0,429		
Progressive Motility (%)	0.071	0.715	−0.089	0.647	−0,016	0,936	−0,170	0,379
Total Motility (%)	0.087	0.655	0.038	0.845	0,031	0,872	−0,041	0,833
Fast velocity (%)	0.109	0.572	−0.030	0.877	0,051	0,792	−0,102	0,600
Medium velocity (%)	0.133	0.491	−0.045	0.815	0,113	0,560	−0,117	0,547
Slow velocity (%)	−0.038	0.843	0.020	0.917	−0,130	0,501	−0,013	0,946
VCL (μm/s)	−0.085	0.662	−0.055	0.778	−0,076	0,695	−0,125	0,518
VSL (μm/s)	−0.109	0.574	−0.139	0.472	−0,109	0,573	−0,202	0,293
VAP (μm/s)	−0.110	0.569	−0.109	0.572	−0,107	0,580	−0,188	0,329
LIN (%)	−0.158	0.413	−0.214	0.264	−0,150	0,436	−0,289	0,129
STR (%)	−0.169	0.382	−0.253	0.185	−0,139	0,474	−0,324	0,086
WOB (%)	−0.145	0.454	−0.091	0.638	−0,144	0,456	−0,203	0,290
ALH (μm)	0.028	0.887	0.116	0.548	0,015	0,940	0,052	0,788
BCF (Hz)	−0.082	0.671	−0.256	0.181	−0,024	0,900	−0,326	0,084
B								
Viability (%)	0.223	0.246	0.101	0.602	0,133	0,491	0,019	0,924
Membrane lipid disorder								
Live sperm with lipid disorder ($M540^+/YO\text{-}PRO\text{-}1^-$)	−0.254	0.183	−0.217	0.258	−0,183	0,343	−0,293	0,124
Live sperm without lipid disorder ($M540^-/YO\text{-}PRO\text{-}1^-$)	0.123	0.525	0.120	0.535	0,032	0,868	0,041	0,834
Dead sperm with lipid disorder ($M540^+/YO\text{-}PRO\text{-}1^+$)	−0.063	0.745	−0.099	0.609	0,021	0,913	−0,016	0,933
Dead sperm without lipid disorder ($M540^-/YO\text{-}PRO\text{-}1^+$)	0.263	0.168	0.075	0.698	0,233	0,224	0,100	0,606
Intracellular ROS content								
Live sperm with high ROS ($H2DCFDA^+/PI^-$)	0.169	0.431	0.344	0.067	0,082	0,705	0,221	0,251
Live sperm with low ROS ($H2DCFDA^-/PI^-$)	0.246	0.246	−0.013	0.947	0,174	0,416	−0,061	0,752
Dead sperm with high ROS ($H2DCFDA^+/PI^+$)	−0.191	0.372	**−0.492**	**0.007***	−0,218	0,307	**−0,555**	**0,002***
Dead sperm with low ROS ($H2DCFDA^-/PI^+$)	−0.266	0.209	−0.036	0.853	−0,188	0,378	0,029	0,880
Intracellular superoxide content								
Live sperm with high superoxides ($HE^+/YO\text{-}PRO\text{-}1^-$)	0.118	0.542	−0.086	0.658	0,112	0,565	−0,152	0,431
Live sperm with low superoxides ($HE^-/YO\text{-}PRO\text{-}1^-$)	0.140	0.469	−0.364	0.052	0,088	0,649	−0,374	0,057
Dead sperm with high superoxides ($HE^+/YO\text{-}PRO\text{-}1^+$)	−0.266	0.163	0.348	0.065	−0,222	0,247	0,357	0,057
Dead sperm with low superoxides ($HE^-/YO\text{-}PRO\text{-}1^+$)	0.370	0.042	−0.114	0.555	0,446	0,015	−0,098	0,611

(*) Denotes statistically significant correlations (P < 0.01).

we are aware, only one study previously investigated the absolute telomere length in bovine sperm, reporting an average of 15.3 kb [35]. This earlier study was based on the Telomere Restriction Fragment (TRF) method, which relies on the absence of restriction enzyme target sequences in telomeres. While the TRF method separates telomere fragments in a pulsed field gel electrophoresis and then performs Southern blot hybridization to determine absolute telomere length, it has been reported to overestimate that variable, due to the presence of subtelomeric regions [35]. In spite of this, data obtained in the present work through an improved qFISH method applied to decondensed sperm cells confirmed the range of absolute telomere length in bovine sperm, as figures were

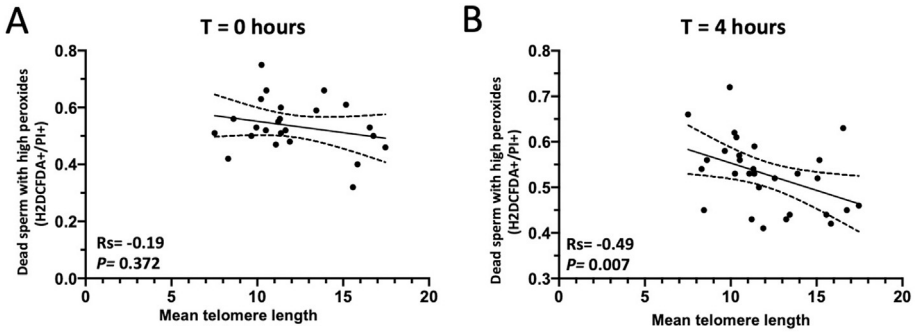

Fig. 3. Correlations between ROS and mean telomere length. (A) and (B) show correlations between telomere length and non-viable sperm with high ROS at 0 h and 4 h post-thawing, respectively.

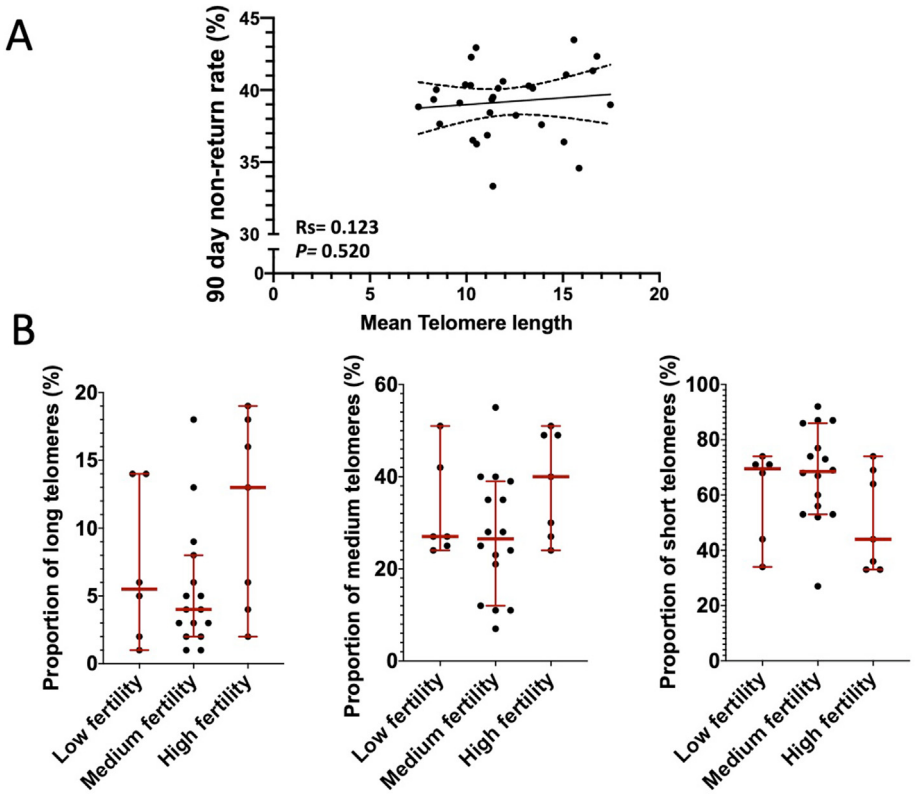

Fig. 4. Sperm telomere length and fertility rates. (A) Shows lack of correlation with 90-day non-return rates. (B) Shows the proportions of long, medium and short telomeres in low, medium and high fertility groups.

similar to those reported previously [35]. Furthermore, the range of telomere length in bovine sperm found herein was similar to that reported in other mammalian species using TRF, such as pigs (between 13 kb and 44 kb) [35,36] and humans (between 10 kb and 20 kb for humans) [35,37–39]. Not only do these results support that telomere length in mammalian sperm is similar across species, but also confirm the robustness of our method (i.e., qFISH in previously decondensed sperm). Comparative studies evaluating sperm telomere length with more than one technique are required to confirm the similarities between species and the suitability of the qFISH technique.

It is worth noting that the use of the qFISH method in this study led us to determine that variation in telomere length within and between sperm cells from the same individual exists, which cannot be observed through qPCR or TRF. Here, we report that the standard variation of telomere length between bovine sperm cells from the same individual was 7.11 kb. Additionally, based on the clustering of individual telomere length, the incidences of each of the three clusters were identified: long telomeres (mean proportion: 7.14%); medium telomeres (mean proportion: 31.03%); and short telomeres (mean proportion 61.93%). Bovine karyotype (2n = 60) consists of 30 chromosome pairs (29 pairs of acrocentric autosomes, and a pair of two submetacentric X chromosomes or a submetacentric X chromosome and a small acrocentric Y chromosome) [40]. This guides one to suggest that most of the short telomeres could belong to the *p* arms of acrocentric chromosomes, which are next to repetitive non-coding centromeric regions and, therefore, do not require large sequences to protect nearby genes. If one assumes that the different chromosome arms may possess distinct telomere lengths, this might lead to two sets of telomeres (shorter and longer). In our study, we observed two predominant subsets of telomeres (medium and short) which could correspond to these two sets of telomeres. Further analyses of qFISH onto metaphase spreads in this species would help establish if differences within distinct chromosome arms exist.

After establishing telomere length in bovine sperm, correlations between this parameter and conventional spermiogram (motility, morphology and viability) and flow cytometry variables (membrane lipid disorder, ROS and superoxide levels) were tested. Evaluations were performed at both 0 h and 4 h post-thawing, in order to assess the resilience of frozen-thawed sperm to incubation at 38 °C. Sperm telomere length did not correlate with motility, morphology or viability after 0 h or 4 h of thawing (Table 1). This contrasts to what reported in a previous study by Iannuzzi et al. [20], who found that sperm motility and DNA damage were different between samples with longer and shorter telomeres. The discrepancies between this study and ours could be explained by the three following differences. First, the study of Iannuzzi et al. [20] was conducted with a lower sample size (n = 16) that included 8 bulls “suitable for reproductive use” and 8 bulls “unsuitable for reproductive use”, without testing correlations between parameters; and animals were aged between 4 and 6 years old. In our work, conversely, we tested correlations, all bulls were suitable for reproductive use, and their age ranged between 1.5 and 2 years old. From studies conducted in other species like humans, the use of a homogeneous group rather a heterogeneous one (fertile and infertile) helps to identify correlations between sperm quality parameters [18,24,25]. Additionally, age may be a factor affecting sperm telomeres, as an increase in sperm telomere length has been suggested with increasing age in humans [41,42], and a raise in the telomere length of offspring might occur when sperm come from older men [43]. Second, we analyzed post-thaw rather than fresh sperm quality. Cryopreservation is known to impair sperm motility and viability, and recent reports in cells other than sperm have

Table 3
Values of sperm quality for each fertility group (low, medium and high fertility). (*) Denotes statistically significant differences compared to the low fertility group ($P<0.01$).

	Low fertility	Medium fertility	High fertility	
	Mean ± Standard deviation	Mean ± Standard deviation	Mean ± Standard deviation	*P*-value (ANOVA Kruskal-Wallis test)
Normal morphology (%)	89.49 ± 3.21	89.12 ± 3.4	88.91 ± 3.87	0.940
Abnormal morphology (%)	10.51 ± 3.21	10.91 ± 3.4	11.09 ± 3.87	0.924
Head abnormalities	0.82 ± 0.97	0.8 ± 0.78	0.78 ± 0.76	0.978
Cytoplasmic droplets	0.16 ± 0.25	0.54 ± 0.8	1.18 ± 0.55	0.010
Tail abnormalities	6.87 ± 2.81	7.19 ± 3.12	5.93 ± 2.81	0.484
Isolated heads	2.68 ± 1.82	2.36 ± 0.98	3.2 ± 2.29	0.846
Total Motility (%)	18.17 ± 4.89	27.74 ± 9.36	33.65 ± 7.88	0.012
Progressive Motility (%)	39.61 ± 8.89	46.21 ± 15.83	55.25 ± 9.57	0.138
Fast velocity (%)	19.99 ± 6.59	30.66 ± 11.16	37.49 ± 11.33	0.030
Medium velocity (%)	10.15 ± 2.41	8.09 ± 2.75	10.1 ± 2.42	0.169
Slow velocity (%)	9.48 ± 7.86	8.66 ± 9.52	7.65 ± 4.39	0.754
VCL (μm/s)	63.38 ± 12.94	85.32 ± 12.26	83.69 ± 22.85	0.032
VSL (μm/s	28.95 ± 8.65	46.36 ± 9.74	45.48 ± 17.04	0.015
VAP (μm/s)	35.88 ± 9.3	52.22 ± 9.54	52.48 ± 18.23	0.023
LIN (%)	45.31 ± 7.61	53.88 ± 4.98	53.02 ± 6.32	0.068
STR (%)	80.07 ± 4.88	88.29 ± 2.83 *	85.85 ± 3.68	**0.003** *
WOB (%)	56.24 ± 6.2	60.91 ± 4.08	61.63 ± 5.67	0.347
ALH (μm)	2.69 ± 0.38	3.25 ± 0.46	2.99 ± 0.54	0.046
BCF (Hz)	10.8 ± 1.64	12.63 ± 0.88	12.39 ± 1.36	0.064
Viability (%)	45.51 ± 8.77	52.97 ± 10.3	52.17 ± 11.79	0.248
Membrane lipid disorder				
Live sperm with lipid disorder ($M540^+$/YO-PRO-1^-)	6.22 ± 2.07	5.58 ± 1.8	5.33 ± 1.05	0.782
Live sperm without lipid disorder ($M540^-$/YO-PRO-1^-)	46.98 ± 8.18	55.33 ± 10.07	55.7 ± 9.79	0.135
Dead sperm with lipid disorder ($M540^+$/YO-PRO-1^+)	46.79 ± 8.9	38.97 ± 10.38	38.9 ± 9.36	0.199
Dead sperm without lipid disorder ($M540^-$/YO-PRO-1^+)	0.03 ± 0.02	0.12 ± 0.16	0.08 ± 0.05	0.133
Intracellular ROS content				
Live sperm with high ROS ($H2DCFDA^+$/PI^-)	4.84 ± 2.51	6.95 ± 6.4	4.81 ± 1.73	0.973
Live sperm with low ROS ($H2DCFDA^-$/PI^-)	38.64 ± 11.1	45.5 ± 10.67	47.8 ± 13.36	0.489
Dead sperm with high ROS ($H2DCFDA^+$/PI^+)	0.53 ± 0.12	0.54 ± 0.06	0.53 ± 0.13	0.949
Dead sperm with low ROS ($H2DCFDA^-$/PI^+)	55.99 ± 9.12	47.01 ± 11.6	46.86 ± 14.18	0.261
Intracellular superoxide content				
Live sperm with high superoxides (HE^+/YO-PRO-1^-)	2.83 ± 0.73	4.35 ± 3.39	4.06 ± 2.25	0.334
Live sperm with low superoxides (HE^-/YO-PRO-1^-)	43.73 ± 8.27	47.25 ± 9.62	50.23 ± 7.73	0.430
Dead sperm with high superoxides (HE^+/YO-PRO-1^+)	48.63 ± 10.14	42.08 ± 9.54	42.89 ± 9.22	0.488
Dead sperm with low superoxides (HE^-/YO-PRO-1^+)	4.82 ± 3.35	6.32 ± 4.52	2.83 ± 1.83	0.169

shown that freeze-thawing may lead to the shortening of telomeres [44,45]. Because whether cryopreservation also contributes to shorten telomeres in sperm is yet to be addressed, we cannot anticipate if the discrepancies between these two studies could be related to the different nature of semen (i.e., fresh or frozen-thawed). Third, while Iannuzzi et al. [20] used the qPCR method, we ran qFISH on previously decondensed sperm. A previous work showed that separate methodologies may provide different telomere lengths in somatic cells [46], but, since sperm have a highly particular chromatin condensation and our modified qFISH method measures telomere length in decondensed chromatin, it is still unknown if different methods could lead to inconsistent results in male gametes.

In physiological processes like capacitation or after being subjected to cytotoxic events, sperm membranes may suffer changes in their fluidity due to the reorganization of membrane lipids [47,48]. In the present work, changes in sperm plasma membrane fluidity have been measured through merocyanine 540, a lipophilic dye that stains membranes with higher affinity when phospholipids become disordered and fluidity is increased [49,50]. Sperm telomere length was not found to be correlated to lipid disorder, suggesting that despite telomeres being attached to the plasma membrane from early stages of spermatogenesis to the mature sperm cell, changes in phospholipid architecture do not affect telomere length. The attachment of telomeres, therefore, may not rely on the architecture of the bilayered membrane. In contrast, we observed that sperm telomere length was also shorter in non-viable sperm whose ROS levels increased after 4 h of incubation. This could coincide with those sperm that, having survived testicular and post-testicular oxidative stress, had their telomere length reduced. After ejaculation, these sperm cells could have converted highly reactive oxygen species such as superoxides into hydrogen peroxide thanks to superoxide dismutase (SOD) activity; this increased ROS could also have ultimately led to sperm death through lipid and protein oxidation during the 4-h incubation period. The fact that, at 4 h post-thaw, the percentage of viable sperm with high ROS was not correlated to telomere length could be attributed either to a higher antioxidant capacity of certain sperm cells or to a lower generation of ROS by these cells during the 4-h period. Related to this hypothesis, it is well known that one of the ROS-generated insults is the oxidation of guanine to 8-oxo-2′-deoxyguanosine (8-OHdG), a derivative that ends up with a DNA break [51]. Telomere shortening associated to ROS has been previously described as a mechanism to prevent the perpetuation of cells exposed to a high risk of mutation [52,53]. In fact, since telomeres consist of hexanucleotide repeats containing three

guanines, they could be especially sensitive to this DNA damage mechanism. In spite of this, previous studies conducted in sperm found no relationship between telomere length and ROS [27,31], thus evidencing the need of new studies, the ours being the first one conducted in bovine.

After establishing the relationship between sperm telomere length and reactive oxygen species, we investigated the correlation between sperm telomere length and *in vivo* fertility rates, expressed as non-return to estrus after 90 days of AI; we found no noticeable results. Additionally, sperm telomere length was not seen to differ between the two extreme fertility groups (<25th percentile *vs.* >75th percentile) (Fig. 4). These results are in agreement with a previous report in bulls, where sperm telomere length and *in vitro* fertility were found not to be correlated [20]. A recent study from our group conducted in pigs, however, found that sperm telomere length is related to *in vitro* embryo development [33]. In that study, we hypothesized that pig embryos with impaired telomeres at morula stage could activate telomerase to restore telomere length, as earlier research showed that telomerase activity is increased at this stage [54]. One cannot, nevertheless, exclude the possibility that if samples from subfertile bulls had been included in the study, a correlation between sperm telomere length and fertility rates would have been more apparent.

In the present study, we have been able to assess sperm telomere length in single telomeres through a modified qFISH applied on decondensed sperm cells. In contrast to other studies that used qPCR or TRF, our method revealed inter- and intracellular variability inherent to each sample. Our study, however, is not exempt of limitations. First, although each of the 29 samples used consisted of a pool of three different samples obtained after 5-week intervals, the resulting low number of biological replicates could have led to spurious observations rather than to significantly biological correlations. In order to diminish the impact of this limitation and thus increase the relevance of the results presented herein, we only assumed statistically significant differences when the *P*-value was less than 0.01. Moreover, one should not exclude that other variables such as DNA fragmentation, testicular telomerase activity, total antioxidant capacity, mitochondrial activity or other seminal plasma characteristics could have an impact on telomere attrition.

5. Conclusions

In conclusion, we determined, for the first time and using qFISH, that telomere length in bovine sperm ranges between 8.31 kb and 17.46 kb, and that it is related to ROS production. In spite of this, no relationship between sperm telomere length and fertility rates was observed in the studied cohort, suggesting that the high selection of the best sires could have led to homogeneous reproductive outcomes.

Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be defined as a potential conflict of interest.

Funding

The present study was funded by the Ministry of Science and Innovation, Spain (AGL2017-88329-R and PID2020-113320RB-I00), the Regional Government of Catalonia, Spain (2017-SGR-1229 and 2020-FI-B-00412), the European Commission (H2020 Research and Innovation program under the Marie Skłodowska-Curie grant agreement No 801342; Tecniospring INDUSTRY, TECSPR-19-1-0003), and La Marató de TV3 Foundation (Grant: 214/857-202039).

CRediT authorship contribution statement

Jordi Ribas-Maynou: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Marc Llavanera:** Investigation, Methodology. **Yentel Mateo-Otero:** Investigation, Methodology. **Nicloas Ruiz:** Investigation, Methodology. **Rodrigo Muiño:** Investigation, Methodology. **Sergi Bonet:** Conceptualization, Funding acquisition, Project Administration, Supervision, Writing – review & editing. **Marc Yeste:** Conceptualization, Funding acquisition, Project Administration, Supervision, Writing – review & editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2022.06.025.

References

[1] Pryce JE, Royal MD, Garnsworthy PC, Mao IL. Fertility in the high-producing dairy cow. Livest Prod Sci 2004;86:125–35. https://doi.org/10.1016/S0301-6226(03)00145-3.
[2] Royal MD, Darwash AO, Flint APF, Webb R, Woolliams JA, Lamming GE. Declining fertility in dairy cattle: changes in traditional and endocrine parameters of fertility. Anim Sci 2000;70:487–501. https://doi.org/10.1017/S1357729800051845.
[3] Lucy MC. ADSA foundation scholar award reproductive loss in high-producing dairy cattle: where will it end? J Dairy Sci 2001;84:1277–93. https://doi.org/10.3168/jds.s0022-0302(01)70158-0.
[4] VanRaden PM, Sanders AH, Tooker ME, Miller RH, Norman HD, Kuhn MT, et al. Development of a national genetic evaluation for cow fertility. J Dairy Sci 2004;87:2285–92. https://doi.org/10.3168/jds.S0022-0302(04)70049-1.
[5] Hermas SA, Young CW, Rust JW. Genetic relationships and additive genetic variation of productive and reproductive traits in Guernsey dairy cattle. J Dairy Sci 1987;70:1252–7. https://doi.org/10.3168/JDS.S0022-0302(87)80138-8.
[6] Berry DPP, Friggens NCC, Lucy M, Roche JRR. Milk production and fertility in cattle. Annu Rev Anim Biosci 2016;4:269–90. https://doi.org/10.1146/annurev-animal-021815-111406.
[7] Gutierrez-Reinoso MA, Aponte PM, Garcia-Herreros M. Genomic analysis, progress and future perspectives in dairy cattle selection: a review. Anim an Open Access J from MDPI 2021;11. https://doi.org/10.3390/ani11030599.
[8] Butler ML, Bormann JM, Weaber RL, Grieger DM, Rolf MM. Selection for bull fertility: a review. Transl Anim Sci 2020;4:423–41. https://doi.org/10.1093/tas/txz174.
[9] Braundmeier AG, Miller DJ. The search is on: finding accurate molecular markers of male fertility. J Dairy Sci 2001;84:1915–25. https://doi.org/10.3168/jds.s0022-0302(01)74633-4.
[10] Farrell PB, Presicce GA, Brockett CC, Foote RH. Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship to fertility. Theriogenology 1998;49:871–9. https://doi.org/10.1016/S0093-691X(98)00036-3.
[11] Kathiravan P, Kalatharan J, Karthikeya G, Rengarajan K, Kadirvel G. Objective sperm motion analysis to assess dairy bull fertility using computer-aided system–a review. Reprod Domest Anim 2011;46:165–72. https://doi.org/10.1111/J.1439-0531.2010.01603.X.
[12] DeJarnette JM, Harstine BR, McDonald K, Marshall CE. Commercial application of flow cytometry for evaluating bull sperm. Anim Reprod Sci 2021:106838. https://doi.org/10.1016/j.anireprosci.2021.106838.
[13] Utt MD. Prediction of bull fertility. Anim Reprod Sci 2016;169:37–44. https://doi.org/10.1016/j.anireprosci.2015.12.011.
[14] Narud B, Khezri A, Zeremichael TT, Stenseth E-B, Heringstad B, Johannisson A, et al. Sperm chromatin integrity and DNA methylation in Norwegian Red bulls of contrasting fertility. Mol Reprod Dev 2021;88:187–200. https://doi.org/10.1002/mrd.23461.
[15] Castro LS, Siqueira AFP, Hamilton TRS, Mendes CM, Visintin JA, Assumpção MEOA. Effect of bovine sperm chromatin integrity evaluated using three different methods on in vitro fertility. Theriogenology 2018;107:142–8. https://doi.org/10.1016/j.theriogenology.2017.11.006.
[16] Dogan S, Vargovic P, Oliveira R, Belser LE, Kaya A, Moura A, et al. Sperm protamine-status correlates to the fertility of breeding bulls. Biol Reprod 2015;92:92. https://doi.org/10.1095/biolreprod.114.124255.
[17] Llavanera M, Ribas-Maynou J, Delgado-Bermúdez A, Recuero S, Muiño R, Hidalgo CO, et al. Sperm chromatin condensation as an in vivo fertility biomarker in bulls: a flow cytometry approach. J Anim Sci Biotechnol 2021;12:115. https://doi.org/10.1186/s40104-021-00634-7.
[18] Cariati F, Jaroudi S, Alfarawati S, Raberi A, Alviggi C, Pivonello R, et al. Investigation of sperm telomere length as a potential marker of paternal genome integrity and semen quality. Reprod Biomed Online 2016;33:404–11.

https://doi.org/10.1016/j.rbmo.2016.06.006.
[19] Torra-Massana M, Barragán M, Bellu E, Oliva R, Rodríguez A, Vassena R. Sperm telomere length in donor samples is not related to ICSI outcome. J Assist Reprod Genet 2018;35:649–57. https://doi.org/10.1007/s10815-017-1104-2.
[20] Iannuzzi A, Della Valle G, Russo M, Longobardi V, Albero G, De Canditiis C, et al. Evaluation of bovine sperm telomere length and association with semen quality. Theriogenology 2020;158:227–32. https://doi.org/10.1016/j.theriogenology.2020.09.019.
[21] Reig-Viader R, Garcia-Caldés M, Ruiz-Herrera A. Telomere homeostasis in mammalian germ cells: a review. Chromosoma 2016;125:337–51. https://doi.org/10.1007/S00412-015-0555-4.
[22] Rocca MS, Foresta C, Ferlin A. Telomere length: lights and shadows on their role in human reproduction. Biol Reprod 2019;100:305–17. https://doi.org/10.1093/biolre/ioy208.
[23] Ferlin A, Rampazzo E, Rocca MS, Keppel S, Frigo AC, De Rossi A, et al. In young men sperm telomere length is related to sperm number and parental age. Hum Reprod 2013;28:3370–6. https://doi.org/10.1093/humrep/det392.
[24] Lafuente R, Bosch-Rue E, Ribas-Maynou J, Alvarez J, Brassesco C, Amengual MJJ, et al. Sperm telomere length in motile sperm selection techniques: a qFISH approach. Andrologia 2018;50. https://doi.org/10.1111/and.12840.
[25] Yang Q, Zhao F, Dai S, Zhang N, Zhao W, Bai R, et al. Sperm telomere length is positively associated with the quality of early embryonic development. Hum Reprod 2015;30:1876–81. https://doi.org/10.1093/humrep/dev144.
[26] Amirzadegan M, Sadeghi N, Tavalaee M, Nasr-Esfahani MH. Analysis of leukocyte and sperm telomere length in oligozoospermic men. Andrologia 2021;53. https://doi.org/10.1111/AND.14204.
[27] Thilagavathi J, Kumar M, Mishra SS, Venkatesh S, Kumar R, Dada R. Analysis of sperm telomere length in men with idiopathic infertility. Arch Gynecol Obstet 2013;287:803–7. https://doi.org/10.1007/s00404-012-2632-8.
[28] Berneau SC, Shackleton J, Nevin C, Altakroni B, Papadopoulos G, Horne G, et al. Associations of sperm telomere length with semen parameters, clinical outcomes and lifestyle factors in human normozoospermic samples. Andrology 2020;8:583–93. https://doi.org/10.1111/ANDR.12734.
[29] Darmishonnejad Z, Zarei-Kheirabadi F, Tavalaee M, Zarei-Kheirabadi M, Zohrabi D, Nasr-Esfahani MH. Relationship between sperm telomere length and sperm quality in infertile men. Andrologia 2020;52:e13546. https://doi.org/10.1111/and.13546.
[30] Turner S, Hartshorne GM. Telomere lengths in human pronuclei, oocytes and spermatozoa. Mol Hum Reprod 2013;19:510–8. https://doi.org/10.1093/molehr/gat021.
[31] Lara-Cerrillo S, Gual-Frau J, Benet J, Abad C, Prats J, Amengual MJ, et al. Microsurgical varicocelectomy effect on sperm telomere length, DNA fragmentation and seminal parameters. Hum Fertil (Camb) 2020:1–7. https://doi.org/10.1080/14647273.2019.1711204.
[32] Muiño R, Tamargo C, Hidalgo CO, Peña AI. Identification of sperm subpopulations with defined motility characteristics in ejaculates from Holstein bulls: effects of cryopreservation and between-bull variation. Anim Reprod Sci 2008;109:27–39. https://doi.org/10.1016/j.anireprosci.2007.10.007.
[33] Ribas-Maynou J, Mateo-Otero Y, Sanchez-Quijada M, Recuero S, Delgado-Bermúdez A, Llavanera M, et al. Telomere length in pig sperm is related to in vitro embryo development outcomes. Anim an Open Access J from MDPI 2022;12:204. https://doi.org/10.3390/ANI12020204.
[34] Ribas-Maynou J, Garcia-Bonavila E, Hidalgo CO, Catalán J, Miro J, Yeste M. Species-specific differences in sperm chromatin decondensation between eutherian mammals underlie distinct lysis requirements. Front Cell Dev Biol 2021. https://doi.org/10.3389/fcell.2021.669182.
[35] Kozik A, Bradbury EM, Zalensky A. Increased telomere size in sperm cells of mammals with long terminal (TTAGGG)n arrays. Mol Reprod Dev 1998;51:98–104. https://doi.org/10.1002/(SICI)1098-2795. 199809)51:1<98::AID-MRD12>3.0.CO;2-Q.
[36] Fradiani PA, Ascenzioni F, Lavitrano M, Donini P. Telomeres and telomerase activity in pig tissues. Biochimie 2004;86:7–12. https://doi.org/10.1016/j.biochi.2003.11.009.
[37] Samassekou O, Gadji M, Drouin R, Yan J. Sizing the ends: normal length of human telomeres. Ann Anat 2010;192:284–91. https://doi.org/10.1016/j.aanat.2010.07.005.
[38] Jørgensen PB, Fedder J, Koelvraa S, Graakjaer J. Age-dependence of relative telomere length profiles during spermatogenesis in man. Maturitas 2013;75:380–5. https://doi.org/10.1016/j.maturitas.2013.05.001.
[39] Fice HE, Robaire B. Telomere dynamics throughout spermatogenesis. Genes (Basel) 2019;10. https://doi.org/10.3390/genes10070525.
[40] Womack JE. The bovine genome. Genome Dyn 2006;2:69–78. https://doi.org/10.1159/000095095.
[41] Baird DM, Britt-Compton B, Rowson J, Amso NN, Gregory L, Kipling D. Telomere instability in the male germline. Hum Mol Genet 2006;15:45–51. https://doi.org/10.1093/hmg/ddi424.
[42] Aston KI, Hunt SC, Susser E, Kimura M, Factor-Litvak P, Carrell D, et al. Divergence of sperm and leukocyte age-dependent telomere dynamics: implications for male-driven evolution of telomere length in humans. Mol Hum Reprod 2012;18:517–22. https://doi.org/10.1093/molehr/gas028.
[43] Kimura M, Cherkas LF, Kato BS, Demissie S, Hjelmborg JB, Brimacombe M, et al. Offspring's leukocyte telomere length, paternal age, and telomere elongation in sperm. PLoS Genet 2008;4:e37. https://doi.org/10.1371/journal.pgen.0040037.
[44] Kim B, Ryu KJ, Lee S, Kim T. Changes in telomere length and senescence markers during human ovarian tissue cryopreservation. Sci Rep 2021;11:1–9. https://doi.org/10.1038/s41598-021-81973-3.
[45] Honda S, Weigel A, Hjelmeland LM, Handa JT. Induction of telomere shortening and replicative senescence by cryopreservation. Biochem Biophys Res Commun 2001;282:493–8. https://doi.org/10.1006/bbrc.2001.4585.
[46] Lai TP, Zhang N, Noh J, Mender I, Tedone E, Huang E, et al. A method for measuring the distribution of the shortest telomeres in cells and tissues. Nat Commun 2017;8:1–13. https://doi.org/10.1038/s41467-017-01291-z.
[47] Gadella BM, Harrison RAP. Capacitation induces cyclic adenosine 3',5'-monophosphate-dependent, but apoptosis-unrelated, exposure of aminophospholipids at the apical head plasma membrane of boar sperm cells. Biol Reprod 2002;67:340–50. https://doi.org/10.1095/BIOLREPROD67.1.340.
[48] Baumber J, Meyers SA. Changes in membrane lipid order with capacitation in rhesus macaque (Macaca mulatta) spermatozoa. J Androl 2006;27:578–87. https://doi.org/10.2164/JANDROL.05135.
[49] Williamson P, Mattocks K, Schlegel RA. Merocyanine 540, a fluorescent probe sensitive to lipid packing. Biochim Biophys Acta 1983;732:387–93. https://doi.org/10.1016/0005-2736(83)90055-X.
[50] Langner M, Hui SW. Merocyanine interaction with phosphatidylcholine bilayers. Biochim Biophys Acta 1993;1149:175–9. https://doi.org/10.1016/0005-2736(93)90038-2.
[51] Ribas-Maynou J, Yeste M. Oxidative stress in male infertility: causes, effects in assisted reproductive techniques, and protective support of antioxidants. Biology (Basel) 2020;9. https://doi.org/10.3390/biology9040077.
[52] von Zglinicki T. Oxidative stress shortens telomeres. Trends Biochem Sci 2002;27:339–44. https://doi.org/10.1016/S0968-0004(02)02110-2.
[53] Berby B, Bichara C, Rives-Feraille A, Jumeau F, Di Pizio P, Sétif V, et al. Oxidative stress is associated with telomere interaction impairment and chromatin condensation defects in spermatozoa of infertile males. Antioxidants 2021;10. https://doi.org/10.3390/antiox10040593.
[54] Gilchrist GC, Kurjanowicz P, Mereilles FV, King WA, LaMarre J. Telomere length and telomerase activity in bovine pre-implantation embryos in vitro. Reprod Domest Anim 2015;50:58–67. https://doi.org/10.1111/rda.12449.