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Research article

EFFECT OF AGE ON SPERM MOTILITY, KINEMATICS, AND MORPHOMETRICS IN BRAHMAN CATTLE

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Bull breeding programs use age as a selection criterion. This study explores the effect of Brahman bull age on sperm motility, morphology, morphometrics, and kinematics. The experiment took place on two cattle farms owned by the Costa Rica Institute of Technology. Thirty-one ejaculates were collected via electroejaculation from 13 sexually mature Brahman bulls, classified into groups according to their age ("Adult" > 31 months;" Young" ≤ 31 months). Semen was evaluated for motility, kinematics, morphology and morphometrics with a CASA ISAS®v1 system and concentration was determined using a photometer. The results showed that the sperm of younger bulls ($p<0.05$) exhibited higher curvilinear velocity (VCL), rectilinear velocity (VSL), and average path velocity (VAP). Sperm linearity (LIN), wobble (WOB), and straightness (STR) were higher in adult than young bulls ($p<0.05$). Adult bull sperm head parameters were higher than for young bulls ($p<0.05$). More elliptical and elongate spermatozoa were observed in adult bulls. The sperm mid-piece area was higher in young bulls. The multivariable assessment identifies four clusters, with differences between each group ($p<0.05$). In conclusion, our results show that age influences the quality of the bovine ejaculate and its potential fertility.

Keywords: Beef cattle, semen, semen analysis, morphology, scrotal circumference, spermatozoa, reproduction

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INTRODUCTION

Livestock production efficiency is usually evaluated by pregnancy rates after artificial insemination (AI) or natural mating [1,2]. Low percentages may be because of the cow, or because of the male and his semen quality and quantity [3]. The bull releases millions of sperm into the female's reproductive system. Each sperm possesses unique traits that may impact its ability to fertilize the egg [1,4]. The success of mammalian reproduction depends largely on the sire ability to produce sperm of adequate quantity and quality [5]. Sperm quality in the vast majority of studies relates to spermatozoa motility [6]; however, over the years these analyses have become more robust with the evaluation of cell kinematics [7–9], morphology [10,11], morphometrics [12–14], and DNA fragmentation [15,16]. Analyzing semen bull quality reveals a considerable variability between ejaculates from the same animal as well as within a single ejaculate. Such variations can have consequences for fertility, even for AI centers that apply stringent standards for semen quality [17–19].

Using semen quality for animal selection not only enhances genetic diversity within the herd but also may lead to higher pregnancy rates and lower costs in cattle production [3,20]. In most mammal species sperm quality declines as individuals age, with a more pronounced decrease occurring after peak maturity or reproductive season [21]. Decreased sperm quality directly leads to decreased fertility [20,22,23]. The effect of sire age on sperm quality has been studied in other species such as dogs [24], stallions [25], salmon [26], bucks [27], boars [23], and bulls [1,20,28].

Bull age strongly influences pregnancy rates and calving intervals [29]. Although there has been much research on how age affects bull sperm quality, the majority of such studies have primarily assessed young animals rather than adults [30]. Studies reveal that age limits the organization of cellular chromatin during spermatogenesis, resulting in increased morphological abnormalities at the cellular level, which in turn affects motility [15]. Age is also associated with increased concentrations of reactive oxygen species and consequent spermatozoa membrane degeneration [24].

The data generated by computer-assisted semen analysis (CASA) are problematic to interpret the biological relevance [9], so multivariate analysis has been used to explain sperm cells behavior by grouping them into clusters or subpopulations [18]. Subpopulation analysis has mainly been used to identify patterns in the behavior of sperm kinematic or morphometric variables and relate them to potential fertility [7,31]. Therefore, we evaluated how swimming sperm and kinematics variables, morphology, and morphometrics in Brahman (*Bos indicus*) cattle are affected by age. We have also explored how age influences the spermatozoon clusters and distribution according to kinematic variables with a multivariable assessment.

MATERIAL AND METHODS

Experiment location, animals, and semen collection

The experiment was conducted from May to August 2021 at two cattle farms in San Ramón and San Carlos, Alajuela, Costa Rica owned by the Costa Rica Institute of Technology. It followed all regulations for working with live animals in Costa Rica. Thirteen sexually mature Grey-Brahman bulls were used as semen donors. These farms typically remove bulls from the reproductive herd after 60 months of age and introduce replacements when they are 24–30 months old. Bulls were classified into groups according to their age. The groups had an average age of 64.19 ± 1.30 months (Adult: > 31 months, n = 6) and 29.47 ± 0.91 months (Young: ≤ 31 months, n = 7). Prior to the experiment bulls had passed a standard breeding soundness evaluation [29], and were sexually abstinent at least seven days before semen collection. Bull scrotal circumference (SC) was measured prior to each semen collection using a tape for this task. At least two semen samples were obtained from each bull; the interval of collection was at least seven days with a maximum of twenty-eight days. A total of 31 ejaculates were collected by electroejaculation using a Lane Pulsator V (Lane Manufacturing, Denver, CO, USA). An automatic program was used with a preprogrammed cycle alternating stimuli of increasing intensity until ejaculation. If the bull did not ejaculate on the first collection attempt, the animal was allowed to rest 5–8 minutes before a second collection was attempted. Semen samples were collected in sterile 15 mL Falcon tubes and the volume was recorded. Next, the samples were diluted in Eppendorf® tubes (Sigma-Aldrich, St. Louis, MO, USA) 1:1 (vol:vol) by one-step with a commercial extender Bioxcell® (IMV, L'Aigle, France) prewarmed at 37 °C to avoid thermal shock. Finally, samples were transported to the laboratory for further analysis.

Motility and kinematic assessment

Sperm motility and kinematics were assessed using a CASA-Mot system ISAS®v1 (Integrated Semen Analysis System, Proiser R+D, Paterna, Spain). The CASA system included a UB203 microscope (UOP/Proiser R+D) with a 1x eyepiece and a 10x negative-phase contrast objective (AN 0.25) equipped with a video camera (Proiser 782M, Proiser R+D) that captured images at 40 frames per second (fps) yielding a final resolution of 768 × 576 pixels. The microscope heated stage was set at 37.0 ± 0.5 °C. Samples of 2.7 µL of previously diluted semen were placed in a Spermtrack® reusable counting chamber (Proiser R+D., Paterna Spain) preheated to 37 °C. Seven visual fields with at least 600 cells in total were analyzed. The system analyzed total motility (TM) and progressive motility (PM). It also considered sperm velocity: rectilinear velocity (VSL, $\mu m.s^{-1}$), curvilinear velocity (VCL, $\mu m.s^{-1}$), average path velocity (VAP, $\mu m.s^{-1}$), the velocity ratios of linearity (LIN = VSL/VCL·100), straightness (STR = VSL/VAP·100), and wobble (WOB = VAP/VCL·100). It also measured sperm

undulation consisting of crossover frequency (BCF, Hz) and amplitude of lateral head displacement (ALH, μm). Finally, sperm concentration of each ejaculate was measured with a Bovine Accuread photometer (IMV, L'Aigle, France).

Sperm morphology and morphometric assessment

Cell morphology was assessed by placing 10 μL of previously diluted and mixed semen on a slide and smeared and covered with a coverslip. Then the sample was introduced into the Trumorph® (Proiser R + D, SL, Paterna, Spain) for fixation as described by Soler et al. [32]. Thereafter samples were observed under a microscope. Cell morphology assessment used 200 cells per sample to calculate the percentage of normal and abnormal cells. Morphometric measurements were taken using two subsamples from each ejaculate. To accomplish this 10 μL of the sample was placed and smeared on two slides. The slides were air dried and stained with Diff-Quik® commercial kit (Medion Diagnostics, Düdingen, Switzerland). To safeguard samples for subsequent analysis, a cover slip was positioned atop each stained slide. Slides were examined using the CASA-Morph, ISAS®v1 system (Proiser R+D) under a microscope using the protocols described by Víquez et al. [13]. To assess the head and mid-piece, 120 cells were randomly chosen in various fields. Cells were rejected if they attached to background particles or other cells that could impede subsequent image processing [12]. When the boundary between sperm head and mid-piece was unclear, we adjusted the analysis factor of the system. When adjustment was not posible, these spermatozoa were eliminated from analysis. The system evaluated five variables related to head size, four to head shape and four to sperm midpiece. The head size variables were length (L, μm), width (W, μm), area (A, μm^2), perimeter (P, μm), and the percentage of the head occupied by the acrosome. Sperm shape was analyzed using the variables ellipticity (L/W), roughness ($4\pi A/P^2$), elongation ([L-W]/[L+W]), and regularity ($\pi LW/4A$), while the cellular midpiece variables analyzed were length (μm), area (μm^2), insertion distance (μm), and insertion angle (°).

Statistical analysis

Data obtained from CASA-mot and CASA-morph analysis of all swimming and kinematics sperm variables and morphometric variables were assessed for homoscedasticity using Levene's tests. Normal probability plot assessed normal distribution. ANOVA further evaluated statistical differences between age treatments. Pairwise comparisons between age means were performed using the Tukey–Kramer test.

Multivariate procedures were performed to identify sperm clusters from this subset of sperm kinematics data. All kinematic variables were standardized to avoid any potential scale effects. A principal factor analysis (PFA) was performed on these data to derive a limited number of linear combinations that maximized the retention of information from the original variables. Prior communalities were estimated based on

the maximum absolute correlation coefficients between each variable and all others. The number of principal factors (PF) to be extracted was determined using the Kaiser criterion, specifically selecting factors with eigenvalues greater than 1. The Kaiser–Meyer–Olkin (KMO) statistic was also calculated to evaluate the dataset's suitability for factor extraction [33]. The varimax rotation method with Kaiser normalization was employed [34]. Correlations between the extracted factors and the original kinematic variables were analyzed to enhance factor interpretability.

Additionally, a clustering analysis categorized spermatozoa into a reduced number of clusters based on factor analysis scores. This analysis was executed in two phases, integrating both hierarchical and non-hierarchical clustering techniques. Initially, factor scores for all spermatozoa were subjected to hierarchical clustering using the Ward Minimum Variance method [35]. Subsequently, the optimal number of clusters identified was applied as the target for a non-hierarchical K-means clustering analysis [36].

ANOVA further evaluated statistical differences between clusters for all kinematic variables, with a threshold for significance of $p<0.05$. Pairwise comparisons of cluster means were performed using the Tukey–Kramer test. Results were presented as mean ± standard deviation. Additionally, the sperm clusters identified after the clustering procedures for each group were analyzed. The percentage of sperm cells in each age cluster was compared using $\chi 2$-test procedures with a significance level of $p<0.05$. All analyses used IBM SPSS statistical software, version 23.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Adult bulls had higher SC and ejaculation volume ($p<0.05$). No statistical differences were observed in sperm morphology (normal and abnormal), sperm concentration, TM and PM; adult bulls, however, had higher means (Table 1).

Table 1. Bull scrotal circumference and ejaculate characteristics (mean ± SEM) according to each age group.

Variable	Adult	Young
SC (cm)	41.48 ± 0.79^{a}	36.94 ± 0.63^{b}
Ejaculate volume (ml)	8.03 ± 1.03^{a}	4.27 ± 0.73^{b}
Concentration (109 cells/ml)	1.17 ± 0.08	1.08 ± 0.07
Normal sperm (%)	88.40 ± 3.32	87.21 ± 2.45
Abnormal sperm (%)	11.52 ± 1.19	12.74 ± 0.95
TM (%)	74.08 ± 3.48	72.89 ± 2.77
PM (%)	66.29 ± 3.55	63.29 ± 2.82

Abbreviations: **SEM** = standard error of mean; Number of ejaculates (31): Adult (n=12); Young (n=19); **SC** = scrotal circumference; **TM** = total motility; **PM** = progressive motility. **[a-b]** Means with different letters in the same row differ statistically at $p<0.05$.

Table 2 describes bull sperm kinematics values according to age. Younger bulls had significantly higher means for sperm velocity parameters VCL, VSL, and VAP ($p<0.05$). Sperm LIN, WOB, and STR showed a significantly higher mean in adults versus young bulls ($p<0.05$). There was an age effect on one sperm oscillation parameter. ALH and BCF means in younger bulls were significantly higher ($p<0.05$) than for adult bulls.

Table 2. Sperm kinematic variables (mean ± SEM) according to each age group of Brahman bulls.

Variable	Adult	Young
VCL (μm.s^{-1})	73.39 ± 1.39^{b}	82.24 ± 1.08^{a}
VSL (μm.s^{-1})	38.01 ± 1.02^{b}	41. 37 ± 0.79^{a}
VAP (μm.s^{-1})	49.74 ± 1.06^{b}	55.27 ± 0.82^{a}
LIN (%)	48.91 ± 0.30	48.64 ± 0.30
STR (%)	70.87 ± 0.22^{a}	70.00 ± 0.17^{b}
WOB (%)	66.03 ± 0.37	66.02 ± 0.29
ALH (μm)	1.87 ± 0.03^{b}	2.09 ± 0.02^{a}
BCF (Hz)	9.07 ± 0.13	9.08 ± 0.10

Abbreviations: **SEM** = standard error of mean; Number of ejaculates (31): Adult (n=12); Young (n=19); **VCL** = curvilinear velocity; **VSL** = straight-line velocity; **VAP** = average path velocity; **LIN** = linearity of forward progression; **STR** = straightness; **WOB** = wobble; **ALH** = amplitude of lateral head displacement; **BCF** = beat-cross frequency. **^{a,b}** Means with different letters in the same row differ statistically at $p<0.05$.

In adult bulls, sperm head size parameters were higher ($p<0.05$). The sperm head shape was also different between groups, with higher rugosity and regularity in young bulls and ellipticity and elongation in adults ($p<0.05$). On the other hand, sperm mid-piece parameters showed no differences for width, insertion distance, and angle. Sperm mid-piece area, however, revealed significant differences ($p<0.05$), higher for young than for adult bulls (Table 3).

The PFA classified the kinematic parameters into two PF factors for each age group. Adult animals PF_1 were associated positively with VCL, VSL, VAP, LIN, STR, and WOB; and PF_2 were negatively related to LIN and WOB and positively for ALH. Both PFs explained 79.31% of total variance. Young animals PF_1 were associated positively with VCL, VSL, VAP, LIN, and STR, while PF_2 were positively related to VCL and ALH, and also negatively correlated with LIN and WOB. The two components explained 78.72% of total variance (Table 4).

Table 3. Morphometrics variables (mean ± SEM) of sperm head and mid piece according to each age group of Brahman bulls.

Variable	Adult	Young
Sperm head size		
Length (µm)	9.41 ± 0.01[a]	9.19 ± 0.01[b]
Width (µm)	4.97 ± 0.01[a]	4.94 ± 0.004[b]
Area (µm^2)	41.09 ± 0.06[a]	39.77 ± 0.05[b]
Perimeter (µm)	26.25 ± 0.03[a]	25.72 ± 0.02[b]
Acrosome (%)	59.67 ± 0.09[a]	59.29 ± 0.07[b]
Sperm head form		
Ellipticity	1.90 ± 0.003[a]	1.86 ± 0.002[b]
Rugosity	0.75 ± 0.001[b]	0.76 ± 0.001[a]
Elongation	0.81 ± 0.001[a]	0.30 ± 0.001[b]
Regularity	0.89 ± 0.001[b]	0.90 ± 0.001[a]
Sperm mid piece		
Width (µm)	1.90 ± 0.01	1.90 ± 0.01
Area (µm^2)	10.69 ± 0.04[b]	10.83 ± 0.05[a]
Insertion distance (µm)	0.30 ± 0.004	0.31 ± 0.003
Insertion angle (°)	6.77 ± 0.12	6.73 ± 0.09

Abbreviations: **SEM**: standard error of the mean; Number of ejaculates (31): Adult (n=12); Young (n=19); **L** = Length; **W** = Width; **A** =Area; **P** = Perimeter; **Ellipticity** = L/W; **Rugosity** = $4\pi A/P^2$; **Elongation** = (L − W)/(L + W); **Regularity** = $\pi LW/4A$.
[a,b] Means with different letter in the same row differ statistically at $p<0.05$.

Table 4. Eigenvectors of principal factor analysis (PFA)* for kinematic variables of bulls' ejaculates according to each age group.

Age	Adult		Young	
Variable	**PF$_1$**	**PF$_2$**	**PF$_1$**	**PF$_2$**
VCL	0.77		0.66	0.72
VSL	0.96		0.96	
VAP	0.91		0.89	
LIN	0.73	-0.65	0.66	-0.72
STR	0.64		0.61	
WOB	0.62	-0.64		-0.71
ALH		0.83		0.88
BCF				
Var Exp	51.45	27.86	44.65	34.07

Abbreviations: **PFA**: principal factor analysis; **Var Exp**: variance explained in each **PF**: Total variance explained for both **PF**: Adult = 79.31 %; **Young** = 78.72 %. *Expresses the more important variables in each PF. Only eigenvectors >0.6 are presented. **VCL** = curvilinear velocity; **VSL** = straight-line velocity; **VAP** = average path velocity; **LIN** = linearity of forward progression; **STR** = straightness; **WOB** = wobble; **ALH** = amplitude of lateral head displacement; **BCF** = beat-cross frequency.

The PFA identified four clusters for each age group (C_1, C_2, C_3, and C_4). There were differences within and between clusters within each group ($p<0.05$), and the distribution was different for each (Table 5). Adult bull spermatozoa C_1 and C_4 had higher speed values. More progressive clusters were observed in C_2, with higher values for LIN and STR. The C_2 and C_4 were also associated with ejaculates whose sperm had a high oscillatory movement, indicated respectively by WOB and BCF; likewise, C_4 also demonstrated the highest value for ALH. In addition, young bull clusters also showed significant differences ($p<0.05$). The C_3 exhibited sperm with higher VCL and ALH. The C_4 contained sperm with higher speed and progressive movement, indicated by VSL, VAP, LIN, and STR. Moreover, spermatozoa with higher oscillatory movement were observed in C_1 and C_4. When each cluster was compared across age groups, differences were found ($p<0.05$). Clusters with higher velocity and progressivity between age groups were C_1 and C_2 in adult bulls and C_3 and C_4 in young bulls (Figure 1).

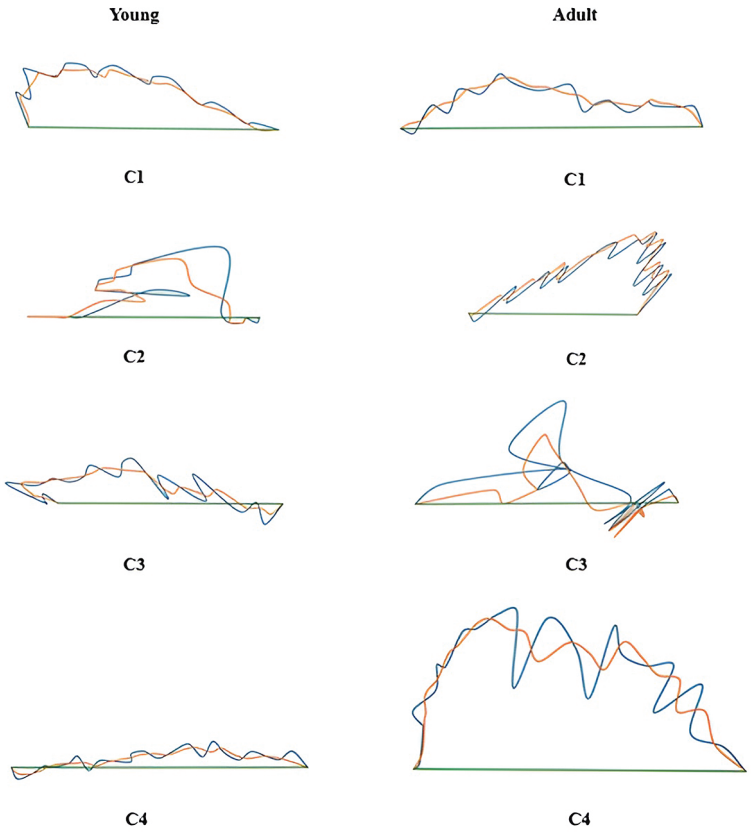

Figure 1. Sperm cluster tracks according to each age group. **Blue line** represents the curvilinear velocity (**VCL**), **orange line** is the average path velocity (**VAP**), and **green line** is the straight-line velocity (**VSL**). **C1** = Cluster 1; **C2** = Cluster 2; **C3** = Cluster 3; **C4** = Cluster 4.

Table 5. Kinematic values (mean ± SEM) of the four sperm cluster according to each age group of Brahman bulls.

Variable	VCL (μm.s^{-1})	VSL (μm.s^{-1})	VAP (μm.s^{-1})	LIN (%)	STR (%)	WOB (%)	ALH (μm)	BCF (Hz)
				Adult				
C1	170.66 ± 0.36$^{b\beta}$	139.91 ± 0.34$^{a\beta}$	143.84 ± 0.32$^{a\beta}$	81.08 ± 0.13$^{b\beta}$	95.27 ± 0.15$^{a\beta}$	83.87 ± 0.11$^{b\beta}$	2.85 ± 0.01$^{b\beta}$	17.36 ± 0.06$^{b\beta}$
C2	98.26 ± 0.37$^{c\beta}$	82.70 ± 0.35$^{c\beta}$	85.04 ± 0.33$^{c\beta}$	82.80 ± 0.14$^{a\beta}$	95.47 ± 0.15$^{a\beta}$	85.52 ± 0.12$^{a\beta}$	1.89 ± 0.01$^{d\gamma}$	13.43 ± 0.06$^{c\beta}$
C3	77.01 ± 0.51$^{d\gamma}$	27.86 ± 0.47$^{d\gamma}$	41.08 ± 0.45$^{d\gamma}$	36.06 ± 0.18$^{d\gamma}$	67.50 ± 0.21$^{c\gamma}$	53.29 ± 0.16$^{d\gamma}$	2.25 ± 0.01$^{c\gamma}$	9.69 ± 0.08$^{d\gamma}$
C4	218.09 ± 0.62$^{a\beta}$	110.94 ± 0.57$^{b\gamma}$	132.37 ± 0.55$^{b\gamma}$	50.55 ± 0.23$^{c\gamma}$	82.52 ± 0.25$^{b\gamma}$	61.22 ± 0.20$^{c\gamma}$	4.48 ± 0.01$^{a\beta}$	19.78 ± 0.10$^{a\beta}$
				Young				
C1	105.83 ± 0.45$^{c\gamma}$	83.62 ± 0.37$^{c\gamma}$	86.05 ± 0.35$^{c\gamma}$	79.55 ± 0.15$^{a\gamma}$	95.83 ± 0.17$^{b\gamma}$	81.93 ± 0.13$^{a\gamma}$	2.07 ± 0.01$^{d\gamma}$	15.50 ± 0.07$^{c\gamma}$
C2	94.67 ± 0.70 $^{d\gamma}$	32.40 ± 0.58$^{d\gamma}$	51.49 ± 0.55$^{d\gamma}$	34.33 ± 0.23$^{c\gamma}$	64.08 ± 0.26$^{d\gamma}$	53.65 ± 0.20$^{d\gamma}$	2.48 ± 0.02$^{c\beta}$	10.82 ± 0.10$^{d\gamma}$
C3	258.91 ± 0.75$^{a\beta}$	115.01 ± 0.62$^{b\beta}$	146.59 ± 0.59$^{b\beta}$	45.37 ± 0.25$^{b\beta}$	77.57 ± 0.28$^{c\beta}$	58.22 ± 0.22$^{c\beta}$	5.46 ± 0.02$^{a\beta}$	18.18 ± 0.11$^{b\beta}$
C4	196.37 ± 0.48^{b}	156.77 ± 0.39 $^{a\beta}$	159.30 ± 0.38$^{a\beta}$	79.71 ± 0.16$^{a\beta}$	96.57 ± 0.18$^{a\beta}$	81.11 ± 0.14$^{b\beta}$	3.19 ± 0.01$^{b\gamma}$	19.12 ± 0.07$^{a\gamma}$

Abbreviations: **SEM** = standard error of mean; n = number of ejaculates; **VCL** = curvilinear velocity; **VSL** = straight-line velocity; **VAP** = average path velocity; **LIN** = linearity of forward progression; **STR** = straightness; **WOB** = wobble; **ALH** = amplitude of lateral head displacement; **BCF** = beat-cross frequency. ^{a,d} Within the same column and age group, means with different letter among sperm clusters differ statistically at $p<0.05$. $^{\beta\ \gamma}$ Within the same column and cluster, means with different letters among age groups differ statistically at. $p<0.05$.

Sperm cluster distribution that correlated with spermatozoa proportion of each cluster showed significant differences and a variable behavior (Figure 2). The clusters with the highest proportion of sperm were C_1 and C_2 in the adult group and C_1 and C_4 in the young group.

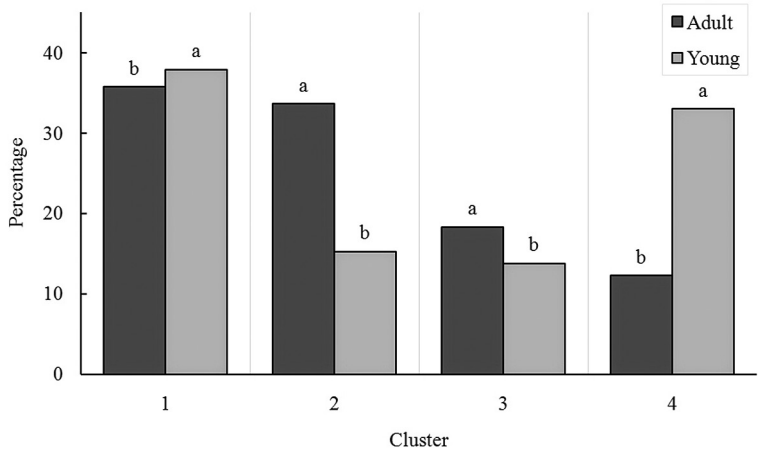

Figure 2. Percentage of sperm cells in each kinematic sperm cluster according to two age groups. **a,b** Different letters superscripts indicate significative differences between cluster at $P < 0.05$ (chi squared ($\chi 2$) test).

DISCUSSION

Our research outcomes on the impact of bull age on semen quality align with earlier investigations. In *Bos taurus*, adult animals produce a higher ejaculate volume than young individuals [1,19,20,28,37]. Likewise, the SC parameter is closely related to animal maturity. It also relates to important semen characteristics such as volume, abnormalities, concentration, total spermatozoa, and cell progressive motility [4,15,29]. In our study, SC produced significant differences between age groups. Sperm abnormalities and progressive movement, however, showed no difference. Moreover, Brito *et al.* [38] reported for *B. taurus*, *B. indicus,* and crossbreeds in Brazil significant increases of ejaculate volume with age, which agrees with our results. The same behavior also has been reported in other domestic species such as pig [39,40], buck [27], ram [41], and stallion [42].

In other species such as the domestic dog, age strongly influences spermatozoon movement by increasing oscillation and linearity and decreasing progressive and total motility [24]. A study by Castro *et al.* [25] found that older stallions have significantly lower MT, MP, and sperm concentration as well as higher morphological abnormalities than young animals. In boars age decreases chromatin stability and sperm morphology, with no effect on progressive motility [23]. In addition, Erraud *et al.* [26] showed that salmon age did not affect motility and kinematics of fresh spermatozoa analyzed

by CASA. Our results partially support that conclusion, because we did not observe differences in TM and PM. We can demonstrate, however, that overall kinematics of young bull spermatozoa exhibit a significantly higher speed and oscillation than those of adults. These results are contrary to other reports in bulls with similar age cohorts [43]. In bulls, the transition from youth to adulthood is marked by a certain stability in sperm motility and morphology [15]. Additionally, once the peak of an animal's reproductive potential is surpassed, sperm motility gradually decreases, thanks to a decline in testicular function and hormonal levels [41]. These factors could in part explain the observed decrease of sperm speed and oscillation in adult animals recorded in this study.

The sperm morphology assessment could identify infertile or sub-fertile bulls [4]; however, our data did not show significant differences between age groups. Analysis of spermatozoan shape and size reflects the importance of semen morphological assessment for bull selection in natural mating or AI programs [10,13]. Our results indicate that adult bull sperm head size, rugosity, and elongation are significantly higher than those of young bulls. A possible explanation could depend on the time of spermatozoan maturity in the epididymis [14]. Because adults may have higher sperm reserves within the epididymis, as well as being more mature, they may have a more developed reproductive tract, which is why the journey of the spermatozoon could take longer than in younger bulls. These results agree with published studies on *B. taurus* [11] and *B. indicus* [16] that the area and width of the spermatozoon's head can be influenced by age; a similar effect has been also reported in boars [23]. Moreover, when analyzing the mid-piece, our study results differ from those reported by Bremer *et al.* [10] for Norwegian Red bulls 10–13 months old. This difference could be related to age and breed. Other studies, nevertheless, have shown that when comparing individuals or conducting multivariate analysis of morphometric variables, the differences become more apparent [44,45]. Furthermore, the results obtained in the present study suggest an important age-dependent relationship between sperm morphometrics and kinematics. The cell head size of mature bulls was significantly larger than in young bulls, even though the presence of large cells could be related to the significant decrease observed in sperm VCL, VSL, and VAP during trajectory. It is interesting that the same effort did not occur on the progressiveness of the movement parameters (LIN and STR). A recent study in *B. taurus* also indicates that sperm cells with small and narrow sizes tend to exhibit higher velocity [13]. In addition, sperm velocity also depends on the sperm ability to move towards the egg, increasing the likelihood of successful fertilization [5].

Our results agree with Peres *et al.* [46], assuming that we can use multivariate analysis to assess potential fertility of a homogeneous population. The PFA and clustering are the best option to analyze large raw datasets from CASA systems [47]; likewise, the distribution of sperm clusters can be influenced by the specific type of statistical multivariate analysis applied [18,31,47]. In our study, eigenvector analysis classified sperm kinematics characteristics into two PFs allowing us to identify four clusters (C_1,

C_2, C_3, and C_4) based on age and variability. The number of kinematic-based clusters in our study is the same as that of other studies on bulls [9,17,18,48–51]. Regarding the analyzed parameters, C_1 for adult bulls showed significantly higher means for VSL, VAP, and STR, describing cell movement as fast, linear, and non-oscillatory with respect to C_2, C_3, and C_4. The C_4 for young bulls showed spermatozoa as fast, linear, and oscillating, with significantly higher means than those of C_1, C_2, and C_3. Several studies indicate that spermatozoa clusters with faster and more linear movement are associated with higher fertility rates [7,18,47,48,52]. We also observed how each cluster showed great variability across age groups. It is interesting in the case of the young group that the greater proportion of sperm cells were in C_1 and C_4, characterizing these as medium to high speed with high progressivity. This information could be relevant for explaining variation between ejaculates and its potential implication for bull fertility. Some studies demonstrated that bull sperm cluster distribution is heterogeneous, because it could be to and individual effect and may be the explanation for differences between ejaculates of the same animal [50]. Furthermore, the observed cluster variations may be attributed to spermatogenesis and the subsequent sperm maturation prior to ejaculation [53]. Apparently, age also shapes the distribution of these clusters. Nonetheless, with all the information explained by the clusters are still not enough to classify an ejaculate based in his quality, partly related to the fact that it is not yet possible to determine which cluster will show the necessary information for ejaculates classifications [18]. For this reason, a comparison of the traditional multivariate and Bayesian analysis has been made to derive a better biological explanation of clusters generated from big datasets provided by the CASA-Mot [9]. In that study, the authors considered that differences between clusters could not be biologically relevant. In part, the uncertainty about the relevance of clustering could be because it is not yet possible to know with certainty which spermatozoon will fertilize the egg and in which cluster it could be located [7].

CONCLUSION

This study showed that the age of Brahman bulls affects sperm quality. We demonstrated that ejaculate volume was higher for adult than young bulls. It also showed that adult bulls produced larger spermatozoa (head and mid-piece) and lower kinematics (VCL, VSL, VAP and ALH) with respect to young bulls. Finally, the multivariable assessment identified multiple clusters with wide variability. Further research is required to better understand the role of age and sperm quality in cattle fertility.

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Ethical statements

The use and care of animals in experimental treatments was in compliance with the Costa Rica Institute of Technology animal welfare guidelines and conducted in accordance with the Three Rs principle. Throughout the study, animals were handled with care to avoid unnecessary stress. This study was performed following ethical principles, and with the approval of the Committee of the Research and Development Center for Sustainable Agriculture in the Humid Tropics of the Costa Rican Institute of Technology (CIDASTH-ITCR), according to Section 22/2022, article 6.0, DAGSC-262-2022, and CIE-470-2022, and this work was part of the research project VIE-5402-2151-1016 "*Protaminas: Evolución y papel en la protección del ADN espermático, formación de la cabeza y funcionamiento celular* (PROTASPERM)". The study was carried out in compliance with ARRIVE guidelines (https://arriveguidelines.org/).

Authors' contributions

AV conceptualize the study. IA-Z, FS, DP-M and AV collected the samples. IA-Z and FS performed the analysis with the software. IA-Z, FS and AV realized the data curation. AV performed the statistical analyses. IA-Z, AV and FS writing the original draft preparation. AV, FS, DP-M, AS-C and BD-M review and editing the manuscript. AV and AS-C project visualization. AV funding acquisition, project supervision and administration. All authors have read and agreed to the published version of the manuscript.

Declaration of conflicting interests

The authors declare that they have no conflicts of interest related to this study.

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UTICAJ STAROSTI NA POKRETLJIVOST, KINEMATIKU I MORFOMETRIJU SPERMATOZOIDA KOD BRAMANSKOG GOVEČETA

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Programi uzgoja bikova koriste starost kao jedan od kriterijuma selekcije. Ova studija istražuje uticaj starosti bika rase braman na pokretljivost, morfologiju, morfometriju i kinematiku spermatozoida. Ogled je sproveden na dve govedarske farme u vlasništvu Tehnološkog instituta Kostarike. Trideset jedan ejakulat je prikupljen putem elektroejakulacije od 13 polno zrelih bikova rase braman, klasifikovanih u grupe prema njihovoj starosti („odrasli“ > 31 mesec; „mladi“ ≤ 31 mesec). Sperma je procenjena na pokretljivost, kinematiku, morfologiju i morfometriju pomoću CASA ISAS®v1 sistema, a koncentracija je određena pomoću fotometra. Rezultati su pokazali da sperma mlađih bikova ($p<0,05$) pokazuje veću krivolinijsko brzinu (VCL), pravolinijsko brzinu (VSL) i prosečnu putanjsku brzinu (VAP). Linearnost spermatozoida (LIN), kolebanje (WOB) i pravolinijski oblik (STR) bili su veći kod odraslih nego kod mladih bikova ($p<0,05$). Parametri glave spermatozoida odraslog bika bili su viši nego kod mladih bikova ($p<0,05$). Kod odraslih bikova primećeno je više eliptičnih i izduženih spermatozoida. Površina srednjeg dela spermatozoida bila je veća kod mladih bikova. Multivarijantna procena identifikuje četiri klastera, sa razlikama između svake grupe ($p<0,05$). Možemo da zaključimo da naši rezultati pokazuju da starost utiče na kvalitet goveđeg ejakulata i njegovu potencijalnu plodnost.