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Bull's age effect on the ratio of X and Y chromosome-bearing spermatozoa

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* Artículo de investigación.

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Abstract

The reproductive efficiency improvements that can be achieved in bovine herds highly depend on the male, as the bulls account for approximately 80% of the gene pool. Likewise, age can impact the sires' reproductive efficiency through its seminal quality, which is strongly influenced by the bull's sexual maturity. The study aimed to assess the bull's age effect on the X and Y chromosomes-bearing spermatozoa ratio in semen. Sixteen Brahman bulls (*Bos indicus*) were grouped into two age range and their reproductive capacity was assessed. Genomic DNA was extracted from a single collection of semen to quantify by qPCR (quantitative polymerase chain reaction) the PLP and SRY genes, used as sexual chromosome markers, and autosomal GAPDH as the reference gene. The values obtained were compared using the delta Ct ($2^{-\Delta Ct}$) method and validated with the use of a commercial sample of semen sexed for the X chromosome. The SRY gene had a proportion of 43% in bulls of 4-5 years of age and 32% in the group of 9-10 years, while the proportions of the PLP gene were 57% in bulls of 4-5 years and 68% in the 9-10 years group. This deviation from the expected ratio (1:1) in bulls of nine and ten years old with a preponderance of the X chromosome content might suggest a possible influence of age in the sex chromosome proportions in the spermatozoa; however, these results were not statistically significant.

Keywords: Bovine sexual chromosomes; X and Y chromosomes ratio; GAPDH; PLP; SRY; Brahman bulls.

Efecto de la edad del toro sobre la proporción de cromosomas X e Y en los espermatozoides

Resumen

Las mejoras en la eficiencia reproductiva que se pueden lograr en los rebaños bovinos dependen en gran medida del macho, ya que los toros representan aproximadamente el 80% del acervo genético. Por otro lado, la edad puede influir en la eficiencia reproductiva de los sementales a través de su calidad seminal, la cual está fuertemente influenciada por la madurez sexual del toro. El objetivo del estudio fue evaluar la influencia de la edad del toro en la proporción de espermatozoides portadores de cromosomas X e Y en el semen. Se agruparon dieciséis toros Brahman (*Bos indicus*) en dos gamas de edad y se

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evaluó su capacidad reproductiva. Se extrajo el ADN genómico de una sola colección de semen para cuantificar por qPCR (reacción en cadena de la polimerasa, cuantitativa) los genes PLP y SRY, utilizados como marcadores de cromosomas sexuales, y el GAPDH autosómico como gen de referencia. Los valores obtenidos se compararon mediante el método delta Ct ($2^{-\Delta Ct}$) y se validaron con el uso de una muestra comercial de semen sexado para el cromosoma X. El gen SRY tuvo una proporción del 43% en toros de 4-5 años de edad y del 32% en el grupo de 9-10 años, mientras que las proporciones del gen PLP fueron del 57% en toros de 4-5 años y del 68% en el grupo de 9-10 años. Esta desviación de la relación esperada (1:1) en toros de nueve y diez años con predominio del contenido del cromosoma X pudiera sugerir una posible influencia de la edad en las proporciones de los cromosomas sexuales en los espermatozoides del semen; sin embargo, estos resultados no fueron estadísticamente significativos.

Palabras clave: cromosomas sexuales bovinos; proporción de cromosomas X e Y; GAPDH; PLP; SRY; toros Brahman.

Introduction

After the introduction of the flow cytometry classification technique to achieve sexual predetermination in semen, multiple tools using biotechnology have been developed to help genetic improvements in the reproduction of cattle (1). The scope of these techniques lies in the commercial interests desired; for example, the preselection of sex plays an outstanding economic role in the breeding programs in the dairy and beef industry, in which female cattle are required in the former whereas male cattle are preferred in the latter (2). Analysis of the sex ratio of spermatozoa is important for the selective breeding of cattle by artificial insemination. Although flow cytometry is a reliable and efficient method, the sorted semen requires reanalysis to quantify its purity using a different technique (3). The qPCR (quantitative polymerase chain reaction) is an efficient and simple tool that combines the enormous sensitivity of PCR with the precise monitoring in real time of its products, allowing it to quantify, e.g., the amount of DNA of sex chromosomes in semen samples (4, 5). On the other hand, all the advances that can be achieved in a herd will depend largely on the male because, in every bovine production unit, the bull is responsible for 80% or more of the genetic improvement (6-9). A bull is considered fertile when it obtains good physical condition, good libido, reproductive capacity, and good seminal quality (10). The reproductive performance of young bulls varies according to the age of onset of puberty; older bulls have greater semen volume and quality; the latter being affected by genetic and environmental factors (11).

Considering that age can influence the reproductive efficiency of the bulls (11), a not studied enough aspect is whether the proportion of X and Y chromosome-bearing spermatozoa in bovine semen changes with the age of the animal. Many factors have been postulated affecting this proportion: season, nutrition, and male ejaculation frequency, among others (11, 12). A recent study showed that males of Holstein bovine with higher testosterone content in the semen had a higher proportion of Y chromosome-bearing spermatozoa, being the testosterone concentration positively correlated with the age of the bull (13). Mice showed different results—there is no evidence that sperm sex ratio variation is driven by testosterone production (14). Some other research has been conducted to assess variation in the ratio of X and Y-bearing spermatozoa in bulls (15), but almost none evaluated the influence of the age of the bull on this ratio. Therefore, this investigation aimed to assess the effect of the age of the bull on the proportion of X and Y chromosomes-bearing sperm, using as a case study the Brahman bulls from the Fernando Corrales herd, in the Apure state, Venezuela.

Material and Methods

Bulls

The herd from which the samples were collected belongs to the Hato Fernando Corrales, from the "Ezequiel Zamora National Experimental University of the Western Plains" (UNELLEZ), located at Mantecal, Bruzual municipality of Apure State, Venezuela.

Bulls were maintained under uniform feeding and management conditions, fed with *Brachiaria brizantha* ad libitum on pasture paddocks, and ad libitum access to water. The sample size was not previously estimated because it is the number of bulls belonging to the commercial cattle farm "Fernando Corrales". It consisted of seven 4-year-old (y. o.), two 5-y.o., two 9-y.o., and five 10-y.o. bulls. A total of 16 Brahman breed bulls were included in the study. For the statistical analysis of the results, the samples were grouped into two age groups, G1: bulls of 4 and 5 years of age (n=9), and G2: bulls of 9 and 10 years of age (n=7).

This work involved the use of non-experimental animals only and followed established internationally recognized high standards of veterinary practice. Ethical approval from a committee was not necessary since the samples were taken by the attending veterinarian during the health monitoring of the bulls.

Collection of Semen Samples

After a 30-day post-service recess period, the semen samples were obtained using the electro-ejaculation method (16), with a prior breeding soundness evaluation (BSE) of each bull, which included physical examination, measurement of scrotal circumference, right and left testicular height, and evaluation of semen quality: volume and density and, spermatozoa quality: mass motility, individual motility, progressive motility, vitality, sperm morphology, sperm concentration, according to standard methodology (17) and the guidelines for bull breeding soundness examinations of the American Society for Theriogenology (18).

A commercial *Bos indicus* sexed semen sample (CSS) for X chromosomes was also acquired, which was used as a control to validate the qPCR technique. Both types of samples were stored at 4ºC for the subsequent extraction of gDNA. Semen sampling was done only once in each animal because many of the bulls were bred to be sold as breeding bulls, which did not guarantee their permanence in the herd.

Extraction, Concentration and Purity of the gDNA

DNA isolation was performed using a combination of the Proteinase K digestion method and subsequent saline extraction (19), according to the protocol of the Human Genetics Laboratory of the Center of Experimental Medicine, at the Venezuelan Institute for Scientific Research (IVIC).

Measurement of gDNA (genomic DNA) concentration was performed with a NanoDrop2000® Spectrophotometer (Thermo Scientific, MA, USA) using 2 µL of the genomic DNA of interest and TE reading buffer (10 mMTris-HCl, 1 mM EDTA, pH 7.0) to remove sample residues at each reading. A Qubit® Fluorometer (Invitrogen, CA, USA) was also used to estimate the DNA concentration by absorbance. The reagents used were from the Quant-it ™ dsDNA Assay Kit, Broad Range (Invitrogen). In order to obtain an average of the DNA concentration of the sample, each reading was performed in duplicate.

Quantitative PCR (qPCR) Assays

Primer design: Specific primers were designed for exon 1 (unique) of the SRY *Bos indicus* gene (selected as a specific marker of the Y chromosome), exon 3 of the PLP *Bos indicus* gene (as a specific marker of the X chromosome), and exon 9 of the autosomal GAPDH *Bos indicus* gene (used as a reference gene). For the design, the free access software Primerblast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and the DNAman software to check the quality parameters of each primer were used. The sequences of the primers and the amplification conditions are shown in Table 1.

Table 1. Primer Sequences and SRY, PLP and GAPDH genes Amplification Conditions

Gene	Primer	Sequence	Product lenght (bp)	Tm (°)
SRY	Forward	5'- CCCATTCTTTGAGGAGGCACA	172	60
	Reverse	5'- CCTGTATGTGAAGGGGTGCAA		
PLP	Forward	5'- ACTACAAGACCACCATCTGCG	192	60
	Reverse	5'-TGTCCCCCACCCCTTGTTA		
GAPDH	Forward	5'-CCAGCCTACCCCATACTCAG	172	60
	Reverse	5'- GCTGCAAACAACTCCTTGGTG		

bp: base pairs; Tm: melting temperature

Amplification by qPCR of the SRY, PLP and GAPDH Genes

To perform qPCR measurements the Eco Real-Time PCR System™ (Illumina, CA, USA) was used, with SYBR Green fluorochrome and GoTaq® qPCR Master Mix (Promega, WI, USA). The final reaction volume for each gene was 10.5 µL, consisting of 10 ng of gDNA, 5 pmol of the corresponding primers, 5 µL (2X) of the commercial SYBR Green GoTaq qPCR Master Mix (Promega); the final volume was adjusted with sterile distilled water.

Each sexual chromosome gene was simultaneously measured with the reference gene in each sample, analyzing it in duplicate or triplicate, and Ct (threshold cycle) values that did not differ more than 0.8 cycles between duplicates/triplicates were considered valid. Melting curve analysis was performed in all cases to verify the specificity of the reaction products. The number of cycles of the PCR reaction was 40, with the mentioned Tm temperature (Table 1). The analysis of the qPCR data was carried out with the Eco Study software version 5.0.4890© (Illumina).

Data Analysis

Quantitative analysis: The comparative Ct method ($2^{-\Delta Ct}$ method) was used to perform the relative quantification analyses of SRY and PLP, as it is a method that normalizes the amplifications of the genes of interest against an internal reference gene (20), in our case, GAPDH.

Statistical analysis: To determine the significance of the results obtained, the Mann-Whitney U test was used (21), which is constructed from the sum of ranks of the samples, as follows:

$$U_1 = n_1 n_2 + \frac{n_1 + (n_1 + 1)}{2} - R_1$$

$$U_2 = n_1 n_2 + \frac{n_2 + (n_2 + 1)}{2} - R_2$$

where n1 and n2 are the sizes of each sample group, and R1 and R2 are the sum of the ranks for groups 1 and 2, respectively. The U statistic is defined as the smaller from U1 and U2. The level of significance was α=0.05.

Results

Samples

The 16 semen samples collected had a volume between 1 and 6 mL depending on the performance of each bull and only in 5 samples there were remains of urine and erythrocytes. The breeding soundness evaluation (BSE) showed a satisfactory physical condition, with >30% sperm motility, >70% normal sperm morphology and, adequate scrotal circumference (based on age) on all the bulls except bull #1 that showed abnormal sperm motility. The results classified all but one bull as a "Satisfactory potential breeder", according to the criterion for bull breeding soundness examinations of the American Society for Theriogenology. Table 2 shows the results of the BSE.

Extraction, Concentration and Purity of the gDNA

All the samples collected, including the commercial sample of semen sexed for the X chromosome, contained a large number of proteins, as is characteristic of this type of sample. Therefore, it was necessary to centrifuge an additional time and/or add additional volumes of lysis buffer to achieve good cell pack separation; in some other instances, additional amounts of proteinase K and NaCl were added to increase protein clearance. The obtained DNA concentrations ranged between 135 ng/µl and 590 ng/µl. All but one (sample # 9) DNA samples had a 260/280 absorbance ratio >> 1.6 (mean = 1.61; mode = 1.8), indicating good DNA recovery and purity in all samples.

Table 2. Bulls' Breeding Soundness Evaluation (BSE)

Bull id	Age (years)	RTH (cm)	LTH (cm)	SC (cm)	Semen volumen (cc)	Semen density	Mass motility	Individual motility %	Progressive motility %	Vitality %	[C] sp/mL x 10^6	SM %
1	9	22	22	34	1	D	<30%	0	0	0	1.200	N
2	9	17	17	36	5.5	D	30-49%	70	80	70	600	N
3	10	18	18	35	5	D	30-49%	80	70	80	500	N
4	10	18	18	34	3.5	D	50-69%	80	80	80	800	N
5	4	16	16	35	2.5	D	50-69%	70	80	80	800	N
6	4	15	15	31	4	D	>70%	90	90	90	1.200	N
7	4	13	13	30	2	D	>70%	90	90	90	1.200	N
8	10	20	20	32	0.8	D	30-49%	70	70	70	500	N
9	4	11	11	31	4	D	>70%	90	90	90	1.200	N
10	10	16	15	36	3	D	50-69%	90	90	90	800	N
11	4	15	15	33	2	D	50-69%	80	80	80	800	N
12	4	16	15	30	6	D	50-69%	80	80	80	800	N
13	5	14	15	33	1.5	D	30-49%	70	70	70	500	N
14	5	16	15	39	4.5	D	>70%	90	90	90	1.200	N
15	4	16	15	36	3.5	D	50-69%	80	80	80	800	N
16	10	16	19	41	6	D	>70%	90	90	90	1.200	N

SC: scrotal circunference; D: dense; SM: sperm morphology; N: normal > 70%
Id: identification number; RTH: Right testicular height; LTH: Left testicular height

qPCR Amplification of the SRY, PLP and GAPDH Genes

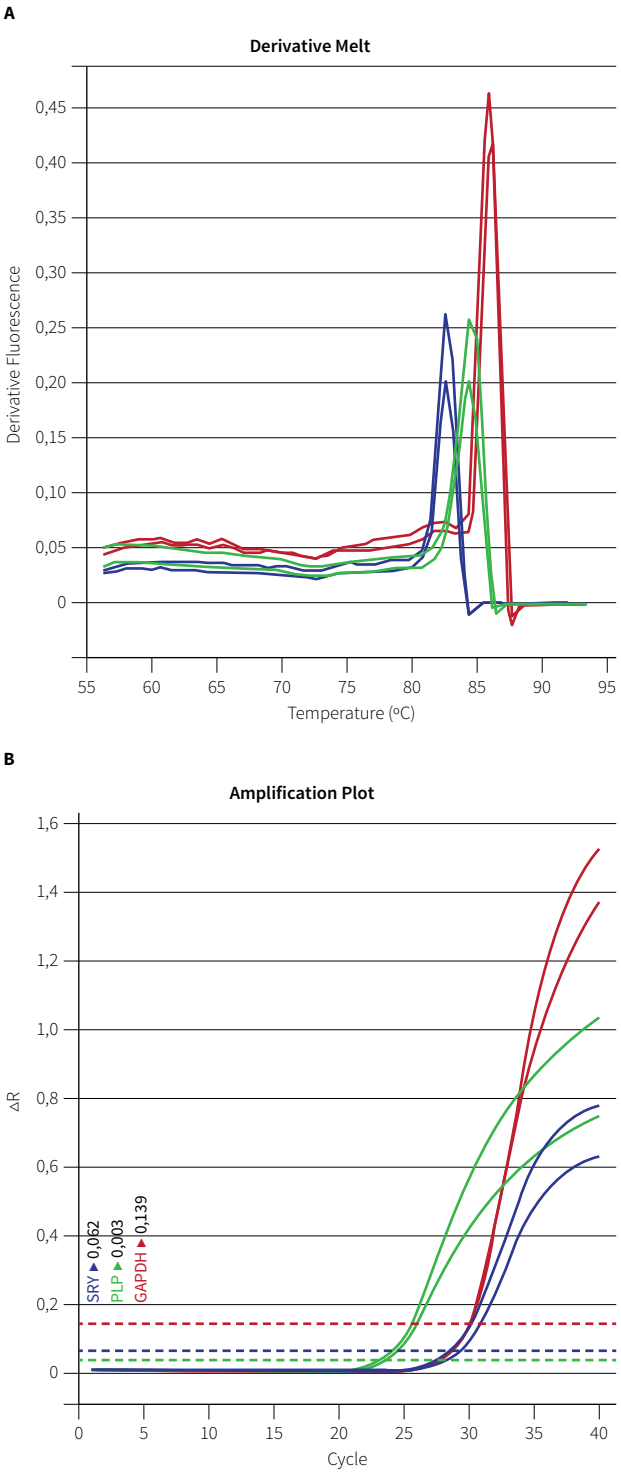
All the studied genes could be detected in all the semen samples, showing adequate and reproducible amplification plots. The melting curves evidenced the presence of specific products without dimer formation or other nonspecific products in the three genes.

The results obtained with the commercial sexed semen sample (CSS) demonstrated the specificity, reliability, and robustness of the detection by the qPCR technique of the 2 sex genes (PLP and SRY) and the autosomal one (GAPDH), as could be observed through the amplification plots; also, the melting curves showed specific products in the three instances (Figure 1A). The Ct values of SRY and GAPDH in the CSS were almost identical and very late (cycle 29) while the Ct value of PLP was obtained in cycle 23, as can be seen in Figure 1B, demonstrating a greater amount of this gene in the sample, as is expected in commercial semen samples enriched in X chromosomes, and very low amount of Y chromosomes (Table 3).

A) The formation of specific products was confirmed by the melting curves. B) The differences between the Ct values for SRY, PLP and GAPDH are evidenced, showing a greater presence of X chromosomes (PLP gene) in this sample, which confirms the specificity of the sample and the qPCR technique. The CSS was sexed for the X chromosome.

Once the specificity of the technique was validated using the sexed semen sample, each gene's average Ct value (PLP and SRY) was normalized by subtracting the Ct value of the internal reference gene (GAPDH), obtaining the ΔCt value for each semen sample, from which the $2^{-\Delta Ct}$ value was calculated to compare the relative amounts of each gene in each sample (Table 3).

Figure 1. Melting Curve (A) and Amplification Plots (B) of the Commercial Sexed Sample (CSS)



Blue line: SRY gene; green line: PLP gene; red line: GAPDH gene.

Table 3. Ct Average Values for Each Gene and Its Relative Quantity Calculated as $2^{-\Delta Ct}$

Sample	Age (y.o.)	Ct SRY	Ct PLP	Ct GAPDH	$2^{-\Delta Ct}$ SRY	$2^{-\Delta Ct}$ PLP
1	9	26.12	23.41	23.11	0.15	0.81
2	9	24.51	24.02	23.52	0.5	0.71
3	10	21.68	22.68	23.11	2.69	1.35
4	10	24.9	24.82	23.81	0.47	0.5
5	4	26.69	25.52	25.0	0.37	0.83
6	4	25.77	24.99	26.17	0.76	0.44
7	4	24.44	24.9	23.46	0.50	0.56
8	10	26.11	23.44	22.38	0.07	0.48
9	4	29.82	30.11	28.64	0.44	0.36
10	10	22.29	21.71	22.53	1.18	1.76
11	4	24.35	20,21	22.08	0.21	0.86
12	4	23.99	23.35	23.37	0.65	1.01
13	5	25.86	22.38	21.42	0.05	0.51
14	5	25.69	25.13	26.15	1.37	0.49
15	4	21.56	21.65	19.13	0.18	0.17
16	10	29.0	22.22	20.46	0.003	0.29
CSS	-	29.05	23.17	29.99	0.019	1.12

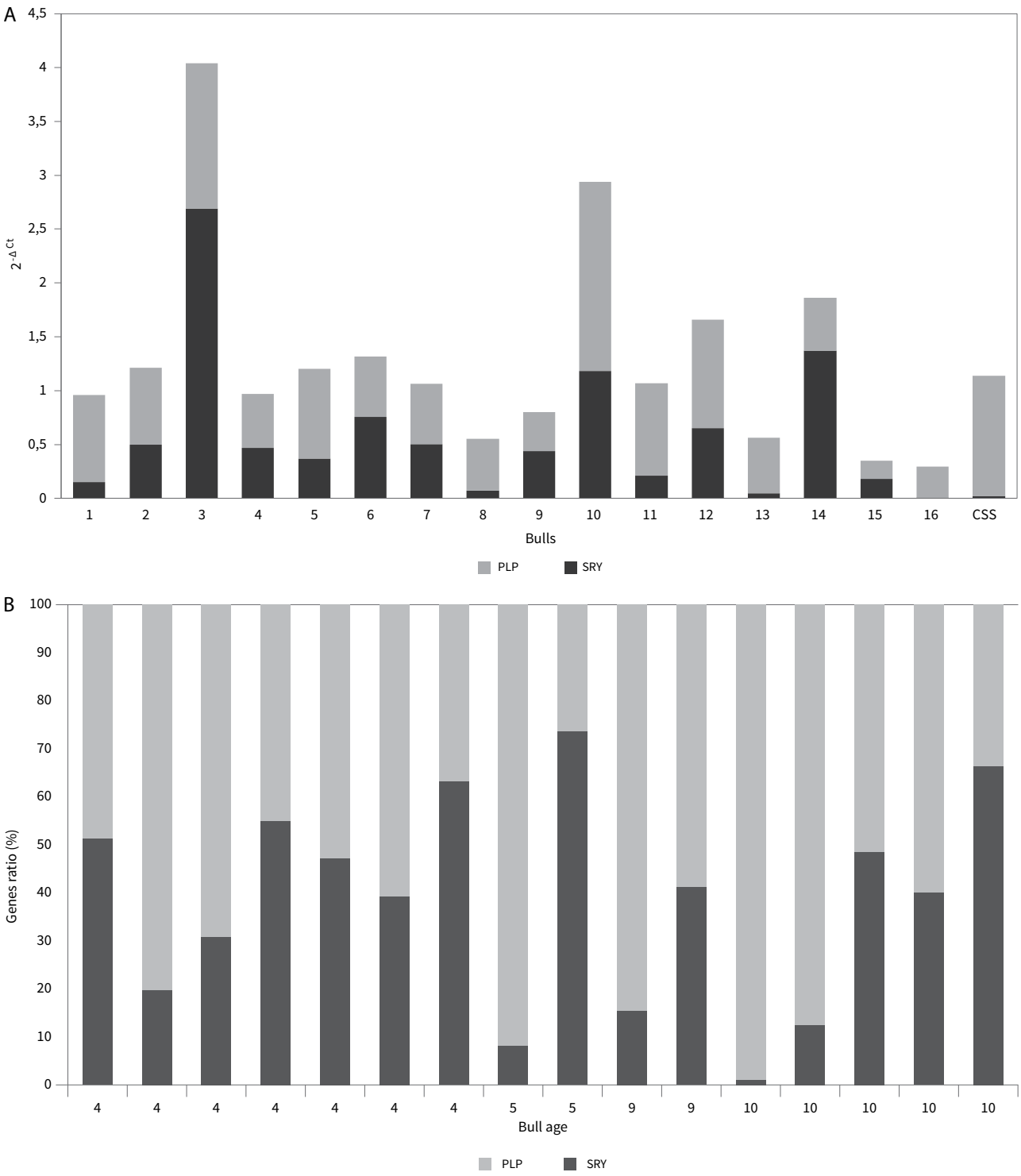
CSS= commercial sexed sample.
Ct values are the average of duplicate or triplicate assays.

A great variability in the relative amount of the genes of sexual chromosomes was observed in the group of bulls; there were a greater number of samples whose sperm had a higher content of X than Y chromosomes (Figure 2A). In addition to the notorious dominance of the presence of the X chromosome, the similarity of results between a 10-year-old individual (sample # 16) and those of the commercial sexed semen sample (enriched in X chromosome content) was remarkable.

When classifying the bulls into two age groups: 4- and 5-year-olds in one group and 9- and 10-year-olds in another group, a clear tendency to a bias in the expected X:Y ratio was noticeable in the older group (Figure 2B).

The proportion of PLP and SRY in each semen sample was calculated, assuming that the sum of the $2^{-\Delta Ct}$ values of each gene in each sample represents the total content of both genes and that each gene represents the

Figure 2. Gene Dosage and Its Ratio in the Semen of the Studied Bulls



A: Relative amounts of each gene according to the $2^{-\Delta Ct}$ values in each animal.
B: Relative proportions (%) of the SRY gene and the PLP gene ordered according to age.
CSS= Commercial sexed sample

total amount of each sex chromosome (X and Y respectively) in the ejaculated semen. The ratio between X and Y chromosomes, expressed as percentages in each sample had a notable variability (Table 4).

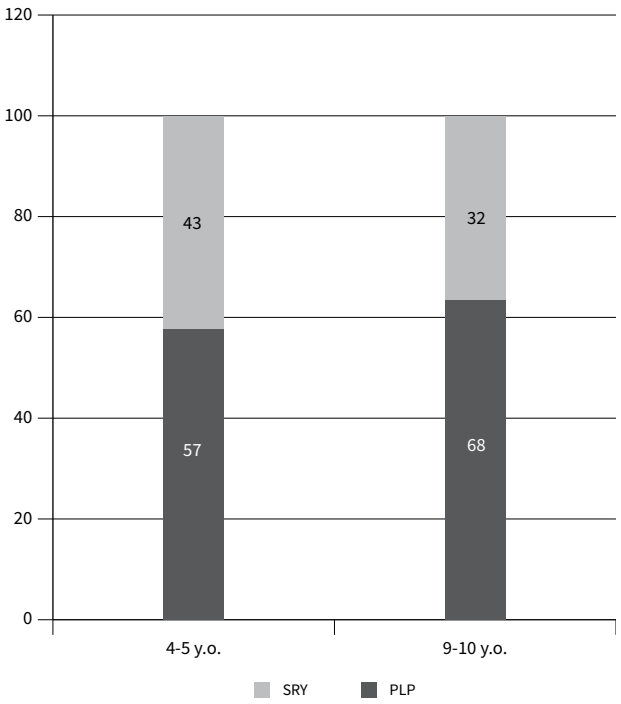
Table 4. Relative Proportions of SRY and PLP Genes in each Semen Samples according to Age

Bull #	Age (y.o.)	$2^{-\Delta Ct}$ SRY	$2^{-\Delta Ct}$ PLP	Total	% SRY	% PLP
15	4	0.18	0.17	0.35	51.42	48.58
11	4	0.21	0.86	1.07	19.62	80.38
5	4	.037	0.83	1.2	30.83	69.17
9	4	0.44	0.36	0.8	55.00	45.00
7	4	0.50	0.56	1.06	47.32	52.68
12	4	0.65	1.01	1.66	39.15	60.85
6	4	0.758	0.441	1.199	63.22	36.78
13	5	0.05	0.51	0.56	8.21	91.78
14	5	1.37	0.49	1.86	73.65	26.35
					$\bar{x}$ = 43.15	$\bar{x}$ = 56.84
1	9	0.15	0.81	0.96	15.62	84.38
2	9	0.5	0.71	1.21	41.32	58.66
16	10	0.003	0.29	0.293	1.02	98.98
8	10	0.07	0.48	0.55	12.72	87.28
4	10	0.47	0.5	0.97	48.45	51.55
10	10	1.18	1.76	2.94	40.13	59.87
3	10	2.69	1.35	4.04	66.58	33.42
					$\bar{x}$ = 32.26	$\bar{x}$ = 67.73
CSS	-	0.019	1.12	1.14	0.02	0.98

CSS= Commercial sexed sample; $\bar{x}$ = arithmetic mean

A tendency to a higher content of X chromosome than Y was observed; it was so in 9 of the 16 samples analyzed (counting those whose difference was greater than 20%). Figure 3 shows the X and Y chromosomes ratio of bulls grouped by age; although both age groups had a higher proportion of X chromosomes (inferred by a higher content of the PLP gene), the older group had it considerably increased. Nonetheless, these differences were not statistically significant, neither for SRY nor for PLP (U= 31.5 and 25.0, $p<0.05$, respectively).

Figure 3. X and Y Chromosomes Ratio (%) according to the Bulls' Age Group



Discussion

The age at which the bull reaches puberty and sexual maturity directly influences seminal quality; 79% of Brahman bulls reach puberty at 18 months with a scrotal circumference between 28 and 29 cm (10). Thus, the age of the bull in which the semen is collected can influence both its quality and the volume of ejaculation (22), these being key factors when selecting future breeders because the semen quality affects its reproductive rates (17). In the present study, after the breeding soundness evaluation (BSE), all but one bull was classified as a "Satisfactory potential breeder" according to the guidelines established by the American Society for Theriogenology (18). Bull #1 had no sperm motility, however, having an adequate sperm count and adequate scrotal circumference.

In bulls the spermatogenesis cycle lasts 61 days; despite the sperm formation begins around day 24 of embryonic development in the yolk sac, the testicles to be

able to produce sperm will have to wait until puberty, when they are sufficiently developed (23). Sperm production is a continuous process, in which the number of chromosomes is reduced by generating haploid gametes (n) from diploid cells (2n). The haploid gametes produced by meiosis must contain a single copy of each chromosome. *Bos indicus* has 60 chromosomes and its gametes 30, with a single sex chromosome (X or Y) in each spermatozoon.

Since sperm production is continuous, many factors can affect it. Injury, illness, fever, and extreme environmental conditions can decrease sperm production. Consequently, it is important to note that the results of a single evaluation of good reproductive status are not valid for the entire life of the bull, and an annual analysis is recommended, usually one month before the start of the breeding season (24). Being the age of the bull an important variable influencing the semen quality, the present study aimed to assess whether old age affects the expected relative proportions (1:1) of the sex chromosomes-bearing spermatozoa in the semen.

The results showed great variability in the content of X and Y chromosomes-bearing spermatozoa in the semen, between bulls (Fig. 2A). Nine out of sixteen animals deviated from the expected ratio (1: 1) with a difference greater than 25% (range of the differences: 26.44 to 97.96%). However, when the results were grouped by age (Fig. 2B), the trend for a higher content of X chromosomes than Y was evident in the 9-10 y.o. bulls, although its group average compared to 4-5 y.o. was not statistically different. The lack of statistical significance could be attributed to the small sample size of each group (n=7 and 9, respectively), and because only one ejaculate was assessed.

Some authors (15) have found that the ratio of X- to Y-bearing sperm differ among bulls, but not among ejaculates within bulls. In contrast, Chandler *et al.* (25) reported little variation among bulls in the ratio of X- to Y-bearing sperm, but differences among ejaculates within bulls. Thus, not only ejaculates but also individual bulls might contribute to an altered sex ratio.

The commercial sample of semen (CSS) sexed for the X chromosome was an excellent quality control for the qPCR technique, as it did indeed show 98% content of this chromosome. The largest deviation from the expected proportions in the bulls was observed in sample # 16 (a ten y.o. bull) whose results were very similar to those of the CSS for the X chromosome. The Ct results obtained for each gene in sample #16 were reproducible and reliable, allowing us to propose that the result was genuine, and not caused by technical artifacts. Rorie et al (15) and Chandler et al (25) found that the range of variation of the percentage of spermatozoa carrying X chromosomes varies between 39 and 75%, following a sinusoidal distribution. This might explain our result. Thus, although the % of the PLP gene of bull #16 is extreme, it was included in the analysis of the results because under natural conditions this variation occurs.

It has been reported that each ejaculate from an animal does not have the same ratio of X and Y chromosomes (25), so the variability observed in the X:Y ratios in the studied bulls seems to be evidence of what happens in natural form. An important limitation of the present investigation is that it was carried out using a single ejaculate from each bull; it is advisable to take several samples per animal leaving intervals of days between each sampling, to avoid biases due to chance. Additionally, a larger number of bulls should be assessed.

The deviations in the expected proportion of X and Y chromosomes (50%:50%) in each evaluated animal can be attributed to several causes, although the information published in this regard is scarce. Among the known ones, possible causes include uneven recombination during male meiosis and subsequent spermatogenesis, seminal quality, semen collection frequency, testosterone, and the influence of seasonal climate. Testosterone concentration decreases with age, and a recent study pointed out its influence on the X and Y-bearing spermatozoa ratio. Bulls with higher testosterone concentrations had significantly higher amounts of Y-chromosome-bearing sperm (13). Therefore, a possible consequence of aging would be a decrease in

the number of Y-bearing sperm and a corresponding increase in the proportion of X-bearing spermatozoa. Our results seem to support those findings, despite the small sample size.

Concerning the meiotic process, there is evidence that the uneven recombination rate of the X and Y chromosomes could influence the survival of the spermatozoa that carry each of them (26). Additionally, during spermatogenesis, a high proportion of malformed spermatozoa are produced, which are lost during the ejaculation (25, 27) contributing to the randomness bias of expected sperm ratios because different ratios of X- or Y-bearing sperm are lost. The findings of different studies on the shape and size of X and Y-bearing spermatozoa and their influence on the X and Y ratio do not support that sperm morphology produces a bias in the expected 1:1 proportions (28).

Regarding the influence of the frequency of ejaculate collection on the variability of the Y chromosome proportions, Chandler et al. (12) suggest as an explanation for this observation that storage in the epididymis would affect it since there is where the final maturation of sperm occurs. The bulls studied in the present work were sexually rested for almost 30 days. Although only one semen collection was carried out, it was made at the end of the post-service recess period. According to Chandler et al. (12), it is at this time that higher variations between ejaculates are reached in the percentage of sperm carrying Y chromosomes in Holstein bulls. In addition to this, collecting samples only once favors the influence of chance on the results obtained, making it difficult to determine if they were attributable to randomness.

Conclusions

The relative amounts and ratios obtained showed an increased presence of X chromosomes in the semen of the 9 and 10-year-old age group; however, these results were not statistically significant. Additional studies are required with a greater number of bulls and more sampling of each animal to elucidate the influence of age on the expected ratio of X and Y chromosomes-bearing spermatozoa in the semen.

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Declarations

Ethics Approval: This work involved the use of non-experimental animals only and followed established internationally recognized high standards of veterinary practice. Ethical approval from a committee was not necessary, since the samples were taken by the attending veterinarian during the health monitoring of the bulls.

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