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INVITED ORIGINAL ARTICLE

Semen Analysis

Computer-assisted sperm morphometry fluorescence-based analysis has potential to determine progeny sex

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This study was designed to determine the ability of computer-assisted sperm morphometry analysis (CASA-Morph) with fluorescence to discriminate between spermatozoa carrying different sex chromosomes from the nuclear morphometrics generated and different statistical procedures in the bovine species. The study was divided into two experiments. The first was to study the morphometric differences between X- and Y-chromosome-bearing spermatozoa (SX and SY, respectively). Spermatozoa from eight bulls were processed to assess simultaneously the sex chromosome by FISH and sperm morphometry by fluorescence-based CASA-Morph. SX cells were larger than SY cells on average ($P < 0.001$) although with important differences between bulls. A simultaneous evaluation of all the measured features by discriminant analysis revealed that nuclear area and average fluorescence intensity were the variables selected by stepwise discriminant function analysis as the best discriminators between SX and SY. In the second experiment, the sperm nuclear morphometric results from CASA-Morph in nonsexed (mixed SX and SY) and sexed (SX) semen samples from four bulls were compared. FISH allowed a successful classification of spermatozoa according to their sex chromosome content. X-sexed spermatozoa displayed a larger size and fluorescence intensity than nonsexed spermatozoa ($P < 0.05$). We conclude that the CASA-Morph fluorescence-based method has the potential to find differences between X- and Y-chromosome-bearing spermatozoa in bovine species although more studies are needed to increase the precision of sex determination by this technique.
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INTRODUCTION

Differences between X- and Y-chromosome-bearing spermatozoa have received great interest from researchers, cattle producers, and the cattle industry in recent decades. The difference in DNA content between spermatozoa with different sex chromosomes is approximately 3.8% in the bull.[1] The use of flow cytometry and sorting enables successful quantification of the DNA content and separation of the two groups of spermatozoa after labeling with Hoechst 33342.[2] However, it is unclear if the differences in sex chromosome content are reflected in differences in sperm head morphometry in the bovine species. Seidel[3] states that nature has gone to extremes to minimize phenotypic differences (for example, in size and shape) between spermatozoa bearing the X- and Y-chromosome (SX and SY, respectively).

van Munster *et al.*[4] described a difference in sperm head volume by micro-interferometry of 3.5%–4% between previously flow-sorted SX and SY in bulls. More recently, Revay *et al.*[5] did not find any size difference between SX and SY after individual sex characterization by FISH. Using atomic force microscopy, Carvalho *et al.*[6] also failed to find differences in any individual morphometric variables (including one-, two-, and three-dimensional parameters and shape descriptors) between SX and SY groups. However, the simultaneous evaluation of all features by the use of statistical discriminant analysis may be a promising method of sperm sexing.[6]

In recent studies, a new automatic method of sperm morphometry assessment combining fluorescence microscopy with image analysis and open-access software (CASA-Morph) has been described.[7] This method reduces the factors with potential effects on morphometric results.[8] In this study, the ability of fluorescence-based CASA-Morph to discriminate between spermatozoa carrying different sex chromosomes from the nuclear morphometric variable values generated and different statistical procedures was tested in bulls.

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MATERIALS AND METHODS

Reagents

Unless otherwise stated, all chemicals used were obtained from Sigma-Aldrich Chemical Company (Alcobendas, Madrid, Spain) and were of the highest grade available.

Semen samples and processing

Animal Care and Use Committee approval was not obtained for this study because no animals were used. Commercial cryopreserved semen samples from 12 Holstein bulls were included in the analysis. Cryopreserved straws were thawed by immersion in a 37°C water bath for 30 s. Semen samples were carefully mixed, and sample aliquots were prepared for fluorescence *in situ* hybridization (FISH), as detailed below, and for sperm morphometry assessment, as previously described.[7]

Fluorescence in situ hybridization (FISH)

Decondensation of spermatozoa and fluorescence *in situ* hybridization was performed as described elsewhere,[9] with slight modifications. Spermatozoa were washed three times in PBS (pH 7.4) containing 6 mmol l^{-1} EDTA and fixed in 3:1 methanol: acetic acid. A 10 μl droplet of the fixed suspension was placed on a clean microscopic slide and air-dried overnight at room temperature. Sperm DNA was denatured by immersion in 3 mol l^{-1} NaOH for 3 min at room temperature followed by short, thorough washing in distilled water and dehydration through a series of ethanol solutions (70, 90, and 100%, v/v) at −20°C for 2 min each and air-dried again.

Metaphase cells for the production of probes through chromosome microdissection were prepared according to standard cytogenetic techniques. For microdissection, a fixed lymphocyte suspension was spread onto a precleaned 24 mm × 60 mm coverslip, which was then air-dried and treated for GTG-banding. The Xcen probe was produced by isolating the pericentromeric region, corresponding to the centromere and with the Xp11-14 region of the standardized GTG-banded karyotype; the probe for chromosome Y was produced from the entire chromosome. Microdissected chromosomes were amplified by following a previously described protocol.[9] Thermal conditions were initial denaturation at 96°C for 3 min, eight cycles performed at 96°C for 1 min, 30°C for 1 min with a 2 min transition from 30°C to 72°C, and 72°C for 2 min. This was followed by 35 cycles of 1 min at 94°C, 1 min at 56°C, and 2 min at 72°C. The final extension was carried out at 72°C for 5 min.

Probes were labeled with digoxigenin-11-dUTP (chromosome Xcen) and biotin-16-dUTP (chromosome Y) (Roche, Mannheim, Germany) in a second Degenerated Oligonucleotide Primer-Polymerase Chain Reaction (DOP-PCR) with 2 μl of products from the first reaction as template. Cycling parameters were 3 min at 95°C for initial denaturation, 30 cycles of 15 s at 94°C, 30 s at 56°C, and 2 min at 72°C, with a 5 min final extension at 72°C.

Probes for the Y-chromosome and for the centromeric region of the X-chromosome of cattle were hybridized simultaneously on metaphase plates for validation and subsequently used for sperm analysis. Probes were precipitated in the presence of 10 μg salmon sperm DNA and 10 μg of calf thymus DNA dissolved in 15 μl hybridization solution (50%, v/v, formamide in 2× standard saline citrate [SSC] + 10%, w/v, dextran sulfate), and finally denatured at 72°C for 10 min, and incubated at 37°C for 60 min.

Metaphase preparations were denatured in 70% (v/v) formamide, 2× SSC (pH 7.0) at 72°C for 3 min (10 min for sperm preparations) and dehydrated through an ethanol series (70%, 85%, 96%, v/v, ethanol, for 2 min each). The hybridization mixture containing probes was applied to the slides and covered with 24 mm × 24 mm coverslips. The slides were hybridized in a moist chamber at 37°C overnight. After hybridization, the slides were washed three times in 50% (v/v) formamide in 2× SSC (pH 7.0) at 42°C for 4 min and three times in 2× SSC (pH 7.0) at 42°C for 4 min. After posthybridization washes, the slides were stained with Hoechst 33342 for sperm morphometric analyses.

The slides were recorded in a setup composed of an epifluorescence microscope (DM4500B, Leica, Wetzlar, Germany, with the appropriate filter sets) with a 63× plan apochromatic objective, and photographed with a Canon Eos 400D digital camera (Canon Inc., Tokyo, Japan). The camera was controlled by a computer through DSLR Remote Pro software (Breeze Systems, Camberley, UK).

Fluorescence imaging and computer-assisted sperm morphometry analyses (CASA-Morph)

Semen smears were allowed to air-dry for a minimum of 2 h, fixed with 2% (v/v) glutaraldehyde in PBS for 3 min, washed thoroughly in distilled water, and labeled with Hoechst 33342. For staining, 20 μl of a Hoechst 33342 suspension (20 μg ml^{-1} in a TRIS-based solution) was placed between the slide and a coverslip, which was then incubated for 20 min in the dark at room temperature.[7] The coverslip was then removed and the slide was washed thoroughly with distilled water and allowed to dry. Fluorescence intensity standards (PS-Speck Blue 360/440, Microscope Image Calibration Kit, Molecular Probes, Madrid, Spain) were mounted on separate slides and used as fluorescence standards.

Digital images of the fluorescence-labeled sperm nuclei were recorded by a setup comprising an epifluorescence microscope (DM4500B, Leica; a-UV filter cube, BP340-380 excitation filter, LP425 suppressor filter, dichromatic mirror: DM400) with a 63× plan apochromatic objective, and photographed with a Canon Eos 400D digital camera. The camera was controlled by a computer through DSLR Remote Pro software.

From each captured image, sperm nuclear morphometry was automatically analyzed by ImageJ open software (available on-line at http://rsbweb.nih.gov/ij/download.html).[7] Each sperm nucleus was measured for four primary parameters and four derived parameters for nuclear shape. Primary parameters were Area (A, μm^2, as the sum of all pixel areas contained within the boundary), Perimeter (P, μm, as the sum of external boundaries), Length (L) and Width (W, μm, the highest and lowest values, respectively, of the Feret diameters, i.e., the projection of the sperm head on the horizontal axis measured at angles of rotation of 0°, 30°, 60°, 90°, 120°, and 150°). Derived nuclear shape parameters were Ellipticity (L/W), Rugosity ($4\pi A/P^2$), Elongation ([L − W]/[L + W]), and Regularity ($\pi LW/4A$). Average fluorescence intensity was also recorded for each sperm nucleus.

Experimental design

Experiment 1

The first trial was to study the morphometric differences between X- and Y-chromosome-bearing spermatozoa. Spermatozoa from eight bulls were processed as explained above for simultaneous assessment of the sex chromosome by FISH and sperm morphometry by CASA-Morph.

Experiment 2

This experiment was to compare the sperm nuclear morphometric results obtained in nonsexed (mixture of SX and SY) and sexed (SX) semen samples from four bulls. Samples were processed for sperm morphometry by CASA-Morph as detailed above.

Statistical analysis

Statistical analyses were performed by using the SPSS package, version 15.0 (SPSS Inc., Chicago, IL, USA). Normality distributions and variance homogeneity of the median value score for each set were checked by the Kolmogorov–Smirnov and Levene tests, respectively.

As all data were normally distributed, parametric tests were used throughout. In Experiment 1, discriminant analysis was performed with the linear stepwise procedure to identify the most useful parameters for the classification of SX and SY spermatozoa. Variables were added one by one to the discriminating functions until the addition of a new variable did not give a better discrimination. Wilk's lambda was used to compare the fraction of the total dispersion of data not accounted for. Both in Experiments 1 and 2, differences in sperm nuclear morphometric parameters between groups were examined through analysis of variance (ANOVA) by using generalized linear models. The values obtained were expressed as mean ± standard error of the mean (s.e.m.). The statistical level of significance was set at $P < 0.05$.

RESULTS

Experiment 1

Figure 1 shows that the successfully sexed spermatozoa were classified according to sex chromosome content as X-bearing (SX) or Y-bearing (SY). **Table 1** contains data referring to the morphometric traits of SX and SY from eight bulls after FISH. These data reveal that SX cells are larger than SY cells on average although with important differences between bulls. The results of ANOVA showed significant differences between the two populations ($P < 0.001$, **Table 1**).

A simultaneous evaluation of all the measured features by discriminant analysis was performed to determine if it was possible to distinguish to which group each individual cell belonged. The results indicated that nuclear area and average fluorescence intensity were the variables selected by the stepwise discriminant function analysis as the best discriminators of SX and SY. The matrix of classification obtained gave the Fisher's discriminant linear functions for each class (**Table 2**, $P < 0.001$). This matrix was applied to the reference population with a globally correct assignment of 54.4% of cells (**Table 3**).

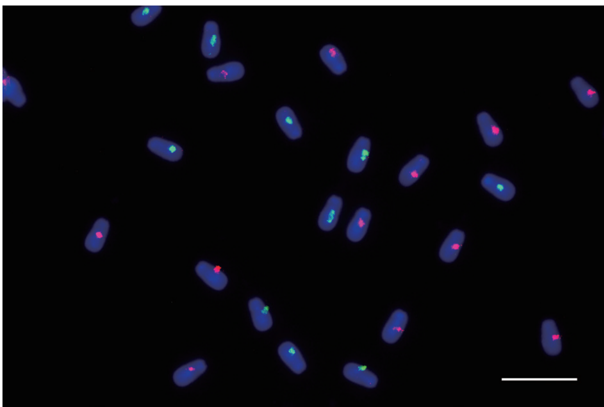

Figure 1: Fluorescence *in situ* hybridization (FISH) with X- and Y-specific probes (X, red; Y, green). Scale bar = 20 µm.

Experiment 2

Results of sperm morphometry for nonsexed and X-sexed semen samples from four different bulls are shown in **Table 4**. Significant differences were found between sexed and nonsexed samples for fluorescence intensity and for all the primary morphometric parameters (A, P, W, and L; $P < 0.05$). X-sexed spermatozoa displayed a larger nuclear size and fluorescence intensity than nonsexed spermatozoa. No statistical differences were found for the secondary morphometric parameters.

DISCUSSION

Sex predetermination of the offspring offers several advantages to the cattle industry such as higher productivity or faster genetic progress among others.[10] Nowadays, selection of sexed spermatozoa before AI is most often used to select the sex of the progeny. Cell sorting by flow cytometry is, to date, the only successful method of separating SX and SY fractions. This method is based on detecting the DNA content differences between SX and SY.[1–3] Methods of detecting a successful separation include flow cytometry, polymerase chain reaction (PCR), and fluorescence *in situ* hybridization.[11] In this study, we evaluated the possibility of using fluorescence-based CASA-Morph for sex determination of bull spermatozoa. The results indicated that this method is able to detect differences in sperm morphometry and

Table 1: Morphometric measurements (mean±s.e.m.) of spermatozoa bearing the X- and Y-chromosome (SX and SY, respectively) from 8 bulls (*n*=200 sperm cells/sample)

Bull	*Sperm*	*Area (µm^2)*	*Fluorescence intensity*	*Perimeter (µm)*	*Length (µm)*	*Width (µm)*	*Ellipticity*	*Rugosity*	*Elongation*	*Regularity*
1	SX	30.59±0.17^a	95.12±1.10^a	23.61±0.072^a	8.72±0.027^a	4.54±0.018^a	1.92±0.008^a	0.69±0.002^a	0.31±0.002^a	1.02±0.002^a
	SY	30.05±0.15^b	93.66±1.12^a	23.39±0.066^b	8.70±0.024^a	4.49±0.017^b	1.94±0.008^a	0.69±0.002^a	0.32±0.002^a	1.02±0.002^a
2	SX	36.46±0.17^a	92.51±0.63^a	25.21±0.056^a	9.07±0.021^a	5.02±0.016^a	1.81±0.006^a	0.72±0.002^a	0.29±0.002^a	0.98±0.001^a
	SY	36.14±0.15^a	92.57±0.49^a	25.00±0.059^b	9.00±0.019^b	5.02±0.017^a	1.80±0.006^a	0.73±0.002^a	0.28±0.002^a	0.98±0.001^a
3	SX	39.07±0.18^a	89.41±0.66^a	25.83±0.059^a	9.31±0.023^a	5.24±0.015^a	1.78±0.005^a	0.74±0.002^a	0.28±0.001^a	0.98±0.001^a
	SY	38.94±0.17^a	88.93±0.65^a	25.87±0.062^a	9.31±0.024^a	5.23±0.016^a	1.78±0.006^a	0.73±0.003^a	0.28±0.002^a	0.98±0.001^b
4	SX	32.87±0.18^a	85.95±0.72^a	24.47±0.068^a	9.08±0.029^a	4.66±0.016^a	1.95±0.008^a	0.69±0.003^a	0.32±0.002^a	1.01±0.002^a
	SY	32.64±0.15^a	84.87±0.72^a	24.43±0.065^a	9.05±0.025^a	4.64±0.015^a	1.96±0.008^a	0.69±0.002^a	0.32±0.002^a	1.01±0.001^a
5	SX	33.70±0.19^a	84.90±0.51^a	24.92±0.071^a	9.31±0.027^a	4.61±0.018^a	2.02±0.008^a	0.68±0.003^a	0.34±0.002^a	1.00±0.002^a
	SY	33.35±0.15^a	83.02±0.48^b	24.84±0.059^a	9.26±0.023^a	4.59±0.017^a	2.03±0.008^a	0.68±0.002^a	0.34±0.002^a	1.00±0.002^b
6	SX	34.25±0.14^a	87.70±0.53^a	24.03±0.048^a	8.80±0.021^a	4.89±0.013^a	1.80±0.005^a	0.74±0.002^a	0.29±0.001^a	0.99±0.001^a
	SY	33.42±0.11^b	86.50±0.49^a	23.85±0.038^b	8.73±0.016^b	4.82±0.013^b	1.81±0.006^a	0.74±0.002^b	0.29±0.001^a	0.99±0.001^a
7	SX	38.37±0.16^a	95.58±0.24^a	26.07±0.059^a	9.45±0.023^a	5.08±0.015^a	1.86±0.006^a	0.71±0.002^a	0.30±0.002^a	0.98±0.002^a
	SY	37.34±0.16^b	94.18±0.27^b	25.73±0.060^b	9.32±0.023^b	5.02±0.016^b	1.86±0.007^a	0.71±0.002^a	0.30±0.002^a	0.98±0.001^a
8	SX	31.45±0.17^a	99.46±1.02^a	23.25±0.058^a	8.60±0.021^a	4.60±0.017^a	1.87±0.006^a	0.73±0.002^a	0.30±0.002^a	0.99±0.001^a
	SY	30.60±0.17^b	97.14±1.13^a	23.03±0.057^b	8.51±0.020^b	4.53±0.017^b	1.88±0.006^a	0.72±0.002^a	0.31±0.001^a	0.99±0.001^b
Mean	SX	34.57±0.10^a	91.55±0.29^a	24.65±0.033^a	9.03±0.011^a	4.83±0.008^a	1.88±0.003^a	0.71±0.000^a	0.30±0.000^a	0.99±0.000
	SY	34.09±0.09^b	89.92±0.28^b	24.54±0.031^b	9.00±0.010^b	4.79±0.008^b	1.88±0.003^a	0.71±0.000^a	0.31±0.000^a	1.00±0.000^b

Different superscripts denote differences between SX and SY within each bull at $P<0.05$. s.e.m.: standard error of mean; SX, SY: spermatozoa bearing the X- and Y-chromosome, respectively

fluorescence intensity between SX and SY although more studies are needed for a successful classification of spermatozoa according to their sex.

The existence of differences in sperm head morphometry between SX and SY in bovine species is controversial.[4–6] Discrepancies might be attributable to the different techniques of sperm sex characterization and sperm morphometric evaluation. In the present study, significant differences in sperm nuclear morphometry between SX and SY were obtained with CASA-Morph after sex determination by FISH. These results contrast with those obtained by Revay *et al*.[5] who observed no significant differences in the head area of SX and SY after FISH. However, the latter authors used conventional fixation-staining techniques, analyzed the morphometry of the whole sperm head, and performed sperm FISH decondensation with papain and dithiothreitol (DTT), not NaOH as here.

The study of sperm nuclear morphometry seems to be more precise than that of the whole sperm head in detecting subtle size differences due to different sex chromosomes. It has been stated that as the mammalian sperm head consists almost entirely of chromatin,[12,13] the morphometric parameters of the sperm nucleus should closely correspond to those of the sperm head.[7] However, the sperm head includes not only the nucleus but also the surrounding plasma membrane and acrosome. The effect of the plasma membrane is minor at best[14] and most likely below the resolution of the imaging system.

Table 2: Discriminant classification matrix showing Fisher's linear discriminant functions

	Coefficient of function of classification	
	SY	*SX*
Area (μm²)	2.40	2.44
Fluorescence intensity	0.676	0.689
Constant	−72.163	−74.441

Values obtained by linear stepwise discriminant analysis. SX, SY: spermatozoa bearing the X- and the Y-chromosome, respectively

Table 3: Percentage of sperm nuclei of the reference population (cells sexed by FISH) assigned to each class by CASMA-F

	Percentage allocated to group by CASMA-F	
	SY	*SX*
FISH-SY	55	45
FISH-SX	46.2	53.8

54.4% of the reference population was classified correctly. FISH: fluorescence *in situ* hybridization; SX, SY: spermatozoa bearing the X- and Y-chromosome, respectively; CASMA-F: computer-assisted sperm morphometry analysis fluorescence

The effect of the acrosome may be more important as it has been demonstrated that the inclusion of the acrosome in head morphometric analysis increases the variability of the results, at least in the ram.[15] This makes the detection of subtle morphometric differences between SX and SY more difficult than if the analysis were focused on the sperm nucleus alone.

The study of sperm morphometry after sex determination by FISH has the limitation that sperm processing, particularly sperm decondensation, causes nuclear swelling, increasing the sperm nucleus/head size in an unpredictable way. However, this effect varies with the method used for sperm decondensation. In this study, the NaOH-based decondensation method was chosen because we had previously observed that the papain-DTT method[5] caused a far larger swelling of sperm heads.

Results of the discriminant analysis indicated that average sperm nuclear area and fluorescence intensity were selected as the best discriminators between SX and SY. The variation in fluorescence intensity after staining with Hoechst 33342 is the basis for separating spermatozoa into X- and Y-chromosome-bearing fractions by flow cytometry.[2,16] Studies comparing the efficiency of flow cytometric and image analysis in tumor diagnosis have concluded that image analysis is just as efficient as flow cytometry, if not better, for distinguishing the DNA content of cells of different ploidies.[11] While the difference between haploid and diploid cells is greater than the difference between X- and Y-chromosome-bearing spermatozoa, the CASA-Morph system detected sperm nuclear area and fluorescence intensity differences between SX and SY in this experiment, even after decondensation.

In Experiment 2, clear differences were found between X-sexed and nonsexed samples for fluorescence intensity and for all the primary morphometric parameters. These differences were greater than those observed between SX and SY in Experiment 1, which may be attributable to the fact that sperm nuclei were not decondensed as occurs in FISH and that the flow cytometer selects a specific SX subpopulation clearly distinguishable by the higher fluorescence intensity of the sperm nucleus.

CONCLUSION

The fluorescence-based CASA-Morph method has the potential to distinguish differences between X- and Y-chromosome-bearing spermatozoa in the bovine species although more studies are needed to increase the precision of sex determination by this technique. These studies should be orientated toward the reduction of the factors other than the gonosomes potentially affecting sperm morphometry, improvements in the equipment and data analysis.

Table 4: Morphometric measurements (mean±s.e.m.) of spermatozoa from nonsexed and X-sexed semen samples (*n*=200 sperm cells/sample)

Bull	*Sample*	*Area (μm²)*	*Fluorescence intensity*	*Perimeter (μm)*	*Length (μm)*	*Width (μm)*	*Ellipticity*	*Rugosity*	*Elongation*	*Regularity*
1	Nonsexed	29.16±0.17[a]	110.37±0.57[a]	22.41±0.063[a]	8.43±0.027[a]	4.35±0.015[a]	1.94±0.007[a]	0.73±0.002[a]	0.32±0.002[a]	0.99±0.001[a]
	X-sexed	32.38±0.13[b]	105.82±0.62[b]	23.44±0.048[b]	8.78±0.021[b]	4.63±0.013[b]	1.90±0.006[a]	0.74±0.001[b]	0.31±0.001[b]	0.99±0.001[a]
2	Nonsexed	36.02±0.15[a]	132.09±0.57[a]	24.60±0.063[a]	9.05±0.02[a]	4.99±0.014[a]	1.82±0.005[a]	0.75±0.003[a]	0.29±0.001[a]	0.99±0.001[a]
	X-sexed	36.14±0.15[a]	120.14±1.50[b]	24.70±0.057[a]	9.20±0.023[b]	4.92±0.013[b]	1.87±0.006[b]	0.74±0.002[a]	0.30±0.002[b]	0.98±0.001[a]
3	Nonsexed	34.88±0.15[a]	122.59±0.97[a]	24.27±0.049[a]	9.04±0.019[a]	4.80±0.014[a]	1.89±0.005[a]	0.74±0.001[a]	0.31±0.001[a]	0.98±0.001[a]
	X-sexed	35.33±0.17[b]	119.63±0.79[b]	24.36±0.059[a]	9.06±0.021[a]	4.84±0.017[a]	1.88±0.005[a]	0.75±0.002[a]	0.30±0.001[a]	0.98±0.001[a]
4	Nonsexed	30.36±0.18[a]	82.08±0.98[a]	23.22±0.066[a]	8.75±0.026[a]	4.35±0.017[a]	2.01±0.008[a]	0.71±0.002[a]	0.34±0.002[a]	0.99±0.001[a]
	X-sexed	33.74±0.13[b]	104.37±0.61[b]	23.86±0.059[b]	8.93±0.023[b]	4.74±0.013[b]	1.88±0.006[b]	0.75±0.002[b]	0.31±0.001[b]	0.99±0.001[b]
Mean	Nonsexed	32.61±0.13[a]	111.79±0.79[a]	23.62±0.043[a]	8.82±0.015[a]	4.62±0.012[a]	1.91±0.004[a]	0.73±0.001[a]	0.31±0.001[a]	0.98±0.015[a]
	X-sexed	34.40±0.09[b]	112.49±0.54[a]	24.09±0.033[b]	8.99±0.012[b]	4.78±0.008[b]	1.88±0.003[b]	0.74±0.001[b]	0.31±0.001[b]	0.98±0.001[a]

Different superscripts denote differences between sexed and nonsexed semen samples within each bull at $P<0.05$. s.e.m.: standard error of mean

AUTHOR CONTRIBUTIONS

PS and JLY conceived and designed the experiments; APa, MAS, SVF, LV, APi, JV, and ES performed the experiments; PS and JLY analyzed the data; PS wrote the paper.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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