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INVITED ORIGINAL ARTICLE

Semen Analysis

Normozoospermic versus teratozoospermic domestic cats: differential testicular volume, sperm morphometry, and subpopulation structure during epididymal maturation

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Teratozoospermia (<40% morphologically normal spermatozoa/ejaculate) is a frequent phenomenon in feline species. This research was carried out to study the possible differences in testicular volume, differential sperm morphometric traits, and potential differences regarding the sperm subpopulational structure during epididymal sperm maturation in teratozoospermic feline donors. Epididymal sperm samples were collected from the caput (R1), corpus (R2), and cauda (R3) epididymidis in two donor groups (N: normozoospermic; T: teratozoospermic). Aliquots were assessed for concentration, viability, motility, and acrosomal integrity. Sperm morphometric descriptors from CASA-Morph analysis were analyzed by the Principal Component Analysis (PCA) and clustering analyses. Irrespective of the group analyzed, PCA revealed two Principal Components (PCs) for each epididymal region explaining more than the 93% of the variance. Surprisingly, the number of subpopulations remained constant in regions R1-R2-R3 irrespective of the donor group analyzed. However, the distribution of these subpopulations was found to be structurally different and strongly influenced by the epididymal region and the donor group. In conclusion, testicular morphometry and the sperm subpopulation structure were different in N and T donors. The alterations in subpopulations during epididymal maturation could be used as a potential clinical indicator of teratozoospermic individuals since an important influence of teratozoospermia on sperm subpopulation structure has been demonstrated.

INTRODUCTION

Most feline species have been categorized by the International Union for Conservation of Nature (IUCN) as threatened. An exception is the domestic cat (*Felis catus*), which has been widely considered the main research model for the study of the reproductive biology to preserve the biodiversity in threatened feline species.[1] The recovery of spermatozoa from the epididymides obtained after orchidectomy or from dead individuals (postmortem) could be a valuable option to preserve potentially valuable genetic material for maintaining germplasm banks.[2] This is of especial interest when considering that spermatozoa collected from epididymides are able to fertilize oocytes.[3,4] The epididymis is an organ in which spermatozoa mature after complex secretory and absorptive processes. Sperm characteristics such as motility and morphology are defined after several stages during maturation, in which plasma membrane changes of sperm cells occur. After this complex maturation process, which involves chemical and physical alterations from the caput and through the corpus epididymidis, the spermatozoa reach the cauda epididymidis where they will be stored before ejaculation. Under normal physiological conditions, the morphological sperm changes that take place during passage through the epididymal duct primarily involve modifications to the sperm head, with final adjustment of sperm dimension and shape.[5,6]

In cats, a high proportion of morphologically abnormal spermatozoa is frequently observed in ejaculates from both domestic and wild species, with individuals considered as teratozoospermic when >60% of cells in the ejaculate are abnormal.[7] However, the causes and mechanisms involved in teratozoospermia are poorly understood and may be caused by membrane modifications during epididymal maturation that influence membrane fluidity that could increase susceptibility to irreversible membrane damage.[8] These aberrant sperm forms have reproductive relevance and have been associated with defects in the acrosome and nucleus.[9] Thus, these suboptimal sperm cells are incapable of penetrating the inner layer of the domestic cat zona pellucida[9] and may ultimately result in infertility.[10] There is strong evidence suggesting that a reduction in genetic diversity (inbreeding) contributes to the establishment of teratozoospermia, impairing spermatogenic function.[11,12] However, it has also been suggested that the causes of teratozoospermia are related to both endogenous (genetics and sexual hormone levels) and exogenous (environment, nutrition, and sexual activity) factors, or even related to stress level and health status.[13,14]

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Computer-Assisted Semen Analysis for Morphology (CASA-Morph) is widely used in veterinary andrology for clinical and research purposes, and it has proven to be a useful diagnostic tool, providing objective analysis of sperm morphometry and setting quality control standards for detecting infertility.[15–17] Thus, physiological assessment of the sperm maturation process requires the critical detection of the slightest alterations in sperm morphometry where CASA-Morph is a relevant and useful system. Moreover, this technique combined with multivariate statistical analysis opens new research possibilities increasing andrological knowledge by identifying sperm subpopulations.[18] Even though sperm morphological studies have been carried out in several species, there are no reports describing the benefits of combining both techniques in feline species. This fact reinforces the idea of using the domestic cat as a model system for threatened felids owing to there being no published studies, to our knowledge, showing objective changes in feline sperm morphometry during sperm maturation in the epididymal duct and their impact on the distribution of sperm subpopulations in teratozoospermic individuals.

Thus, because of this lack of knowledge, the aims of the present research were, in normozoospermic and teratozoospermic domestic cats, to (i) study the possible changes in testicular morphometric characteristics; (ii) evaluate the presumed differential sperm morphometric traits during the epididymal sperm maturation process; and (iii) assess the potential differences in the sperm subpopulation structure during epididymal sperm maturation.

MATERIALS AND METHODS

Ethics statement
The current study was carried out under a Project License from the SENESCYT (PROM-CEB-013-2014) and the Ecuadorian Ministry of Education. We confirm that this study was carried out in strict accordance with the recommendations in the legal framework (Animal Welfare Law) currently in place in AGROCALIDAD Ethics Committee (Ministry of Agriculture, Livestock, Aquaculture and Fisheries), which is the named IACUC for all Ecuadorian Public and Private Laboratories and Higher Education Institutions that specifically approved this study. All procedures were performed in accordance to the Ecuadorian Animal Protection Law (COIP Art. 249-2014) and LOBA-2014. Permission was obtained to perform procedures on all privately owned tomcats. All surgery was performed in veterinary clinics under inhalatory anesthesia (isoflurane), and all efforts were made to minimize suffering.

Chemicals and media
Unless stated otherwise, all reagents and media used in the present study were of high purity and were obtained from Sigma-Aldrich Chemical Corporation (St. Louis, MO, USA). All solutions and media were prepared by adjusting the osmolarity and temperature, at pH 7.4 and using sterile deionized water.

Animals, gonadal collection, and sperm recovery
Reproductive tracts were obtained from 12 privately owned clinically healthy tomcats (*F. catus*; Persian breed; age 12 to 48 months; weight 3.0 to 5.5 kg) immediately after routine bilateral orchiectomy over a 3-month period (January to March). Testes with adjacent epididymides and the vasa deferentia were placed in a sterile Ringer solution (lactate-free) at room temperature and transported to the laboratory within 30 min of gonadectomy for further processing. During epididymectomy, blood vessels were removed to avoid contamination of spermatozoa and the epididymides and proximal deferent ducts were dissected from the testis

and connective tissue with size-15 scalpel blades. The caput (Region 1 [R1]: the 10 mm initial segment closest to the testis), corpus (Region 2 [R2]: middle segment), and cauda epididymidis (Region 3 [R3]: the 10 mm final segment closest to the ductus deferens) were dissected with separate forceps and scissors for each region to prevent cross-contamination, removed separately from both testes in a Petri dish (within the same donor), flushed with 1 ml Dulbecco's Phosphate-Buffered Saline (PBS), penicillin 0.01% (w/v), and streptomycin 0.01% (w/v) (pH 7.4, 285 mosmol kg^{-1}) with a 29-gauge needle, squeezed by forceps and finally cut transversally to permit sperm release and their recovery.[3,5] Finally, the recovered sperm samples were placed in a conventional incubator at 38°C for 15 min to allow for movement of the retrieved spermatozoa. Eight normozoospermic (N, >60% morphologically normal spermatozoa) donors and four teratozoospermic (T, <40% morphologically normal spermatozoa) donors were confirmed by repeated prior semen analysis[7] and after morphological sperm assessment recovered from the cauda epididymidis.[5] All donors were maintained under natural photoperiod conditions and provided with commercial dry cat food and water *ad libitum*.

Testicular gonadometric assessment from normozoospermic and teratozoospermic individuals
Immediately after orchidectomy, testicular measurements were made with laboratory calipers in both normozoospermic and teratozoospermic individuals. The epididymis was not included during the testicular morphometric assessment as it had previously been removed from both gonads before biometric evaluation. Length, height, and width of the right and left testis were measured, and the different values were estimated from the formula for an ellipsoid as follows: testicular volume = length × height × width × (π/6). Finally, the volumes for the right and left testes were combined to obtain total testicular volume for each male.

Epididymal semen processing and sperm quality evaluation
The sperm quality was only evaluated in R3 derived samples because in R1/R2 regions contain spermatozoa with low motility and different plasma membrane characteristics.[5] Sperm motility was subjectively assessed by visual estimation. Briefly, aliquots of epididymal spermatozoa diluted in 1 ml Dulbecco's PBS with 4 mg ml^{-1} Bovine Serum Albumin (BSA), fraction V, penicillin 0.01% (w/v) and streptomycin 0.01% (w/v) (pH 7.4) were withdrawn to analyze subjective motility after 10 min incubation at 38°C. Glass slides were prewarmed to 38°C and 7 μl of the sperm suspensions were covered with a 22 mm × 22 mm coverslip. Sperm concentration was determined from undiluted semen by hemocytometer (Bürker counting chamber); thereafter, sperm samples were diluted to give a final concentration of 50 × 10^6 cells ml^{-1}. Analyses were then immediately performed with a phase-contrast Nikon Eclipse E200 microscope (Nikon, Tokyo, Japan). The functional integrity of the feline sperm membrane was evaluated by eosin/nigrosin staining. The acrosomal integrity was assessed on duplicate sperm smears from each epididymal sample in each donor using the method described by Pope *et al.*[19] in both N and T groups. Briefly, 5 μl of diluted spermatozoa was incubated with 5 μl of stain (Rose Bengal and Fast Green FCF, 1% w/v of each) in a 0.5 ml microtube in the dark for 90 to 120 s at room temperature (25°C). Microscope slides were prepared by placing 5 μl of the stained sperm sample on the clear end of a frosted slide and dragging the drop across the slide to create a thin feathered smear (two smears per epididymal sperm sample). Samples were subsequently dried, mounted, and permanently sealed with Permount™ Mounting Media (Fisher Scientific,

Fair Lawn, NJ, USA) under 22 mm × 60 mm coverslips. At least 250 spermatozoa per sample were randomly analyzed per slide.

Computer-Assisted Semen Analysis for Morphology (CASA-Morph)

Two sperm smears were prepared per region (R1, R2, and R3). Sperm smears were stained, air dried, mounted, and permanently sealed with Eukitt® mounting medium (Fluka BioChemika, Buchs SG, Switzerland) under 22 mm × 50 mm coverslips as described by García-Herreros and Leal.[20] Finally, the slides were used for computerized morphometric analysis by using a commercially available system (Motic Corporation, Ltd., Hong Kong, China) equipped with a Nikon Eclipse E200 (Nikon, Tokyo, Japan) microscope with a 100× oil immersion bright-field objective lens. The video signal was acquired by a MotiCam 2000 digital camera (CMOS ½"; Motic Corporation, Ltd., Hong Kong, China) mounted over the microscope and connected to a Pentium P8400 4GB processor, as described Martí et al.[21] The configuration of the computer system included the interface Motic Images Plus 2.0 ML (Motic China Group, Ltd., Hong Kong) imaging analysis software. Digitized images were made up of 1 920 000 pixels (picture elements) and 256 gray levels. At least 250 sperm cells per sample were randomly captured. Data were compiled and stored for further analysis. Only cells that did not overlap with debris or other cells were considered for analysis. The search, capture, and morphometric analysis of all slides was made by the same person. Each sperm head was measured for four primary dimensional parameters (Area [A, μm^2], Perimeter [P, μm], Length [L, μm], and Width [W, μm]) and three head shape-derived parameters (Ellipticity [EL, (L/W)], Elongation [EO, (L − W)/(L + W)], and Rugosity [R, $(4\pi A/P^2)$]). These morphometric descriptors were chosen to provide maximal statistical information with a minimal number of parameters.[22] Measurements of each spermatozoon were saved in Excel© (Microsoft Corporation, Redmond, WA, USA)-compatible database by the software for further analysis.

Statistical analysis

All analyses were performed with the statistical package SPSS version 15.0 for Windows software (SPSS Inc., Chicago, IL, USA). The effect of the donor group on sperm morphometric characteristic and the variation of morphometric characteristics between donor groups were evaluated using the general lineal model (GLM) for repeated measures. The same procedure was used on the sperm concentration, the extent of intact membranes (viability %), motile cells (motility %), and acrosomal integrity (acrosome damage %) to classify the measured spermatozoa. Differences between R1/R2, R1/R3, and R2/R3, allowing for all the morphometric variables, were evaluated using discriminant analysis. The data matrix from all the sperm cells analyzed by the computer-assisted analysis represented more than 30 000 observations (18 334 for the N group [6010, 6745, and 5579 for R1, R2, and R3, respectively] and 12 568 for the T group [4053, 3637, and 4878 for R1, R2, and R3, respectively]), each one defined by the seven morphometric descriptors specified above. To assess the potential differences in sperm subpopulations during epididymal sperm maturation, Principal Component Analysis (PCA) was performed individually for each epididymal region (R1, R2, and R3) for each donor group. The PCA of these data (each variable was weighed with their eigenvectors) was performed to derive a small number of linear combinations (Principal Components, PCs) that retained as much information in the original variables as possible. As a rotation method, the VARIMAX method with Kaiser Normalization was used. The next step was to perform a nonhierarchical analysis using the k-means model, which uses Euclidean distances from the quantitative variables after standardization of the data, so the cluster centers were the means of the observations assigned to each cluster. The multivariate k-means cluster analysis was for classifying the spermatozoa into a reduced number of subpopulations (clusters) according to their morphometric descriptors, as described by García-Herreros and Leal.[23] ANOVA and Chi-squared test procedures were applied to evaluate statistical differences in the distributions of observations (individual spermatozoa) within the donor group and subpopulations (percentages of spermatozoa assigned), and then a GLM procedure was used to determine the effects of each donor group, as well as their variation, on the relative distribution frequency of spermatozoa within the subpopulations. The GLM procedure was also used to evaluate the influence of the two independent variables on the mean morphometric parameters defining the different sperm subpopulations (i.e., the cluster centers). Differences between means were analyzed by Tukey's test. The level of significance was set at $P < 0.05$.

RESULTS

Testicular metrics in normozoospermic and teratozoospermic domestic cats

The mean testicular length, width, height, and volume in the right and left testis are shown in **Table 1** for both N and T groups. Regarding length, width, and height, no significant differences were found between the right testis and left testis within groups ($P > 0.05$). When both N and T groups were compared, statistical differences in partial testicular volume (right and left testis separately) were found between groups ($P < 0.05$). Finally, statistical differences in total testicular volume (right + left) were detected when N and T groups were compared ($P < 0.05$).

Sperm quality parameters in normozoospermic and teratozoospermic individuals

Sperm quality characteristics derived from different donor groups (N and T) in R3 are shown in **Table 2**. In general, except for sperm concentration, our results indicate that no statistical differences were found in sperm quality parameters in normozoospermic and teratozoospermic donors ($P > 0.05$). The number of retrieved spermatozoa in R3 was significantly higher in the T group than in the N group ($P < 0.05$). The percentage of motile spermatozoa in R3 was not significantly higher in samples recovered from the N group than from the T group ($P > 0.05$). For sperm viability in R3, no significant differences were observed between groups ($P > 0.05$). In addition, our results showed no significant differences between the percentage of spermatozoa with an intact acrosome recovered in R3 from the N and T groups ($P > 0.05$).

Sperm morphometric parameters in normozoospermic and teratozoospermic donors

Table 3 presents the sperm morphometric descriptors assessed in both N group and T group from different epididymal regions (R1, R2, and R3). Interestingly, after sperm morphometric analysis, significant differences in sperm dimensions and shape parameters were found among epididymal regions when the N group and T group were compared ($P < 0.01$). Thus, sperm morphometric parameters analyzed from R1, R2, and R3 resulted in significant differences in both dimensions and shape among different epididymal regions within groups ($P < 0.001$). Moreover, statistical differences related to all sperm dimensions (mainly A, P, and L) and shape (mainly ellipticity) were detected in R1, R2, and R3 between groups ($P < 0.001$).

PCA and sperm morphometric data derived from normozoospermic and teratozoospermic individuals

The data matrix consisted of 30 902 observations. PCA rendered two PCs with eigenvalues >1 in each epididymal region (R1, R2, and R3)

Table 1: Testicular metrics (testicular morphometry) in N and T domestic cats

Donor group	Testicle	Testicular metrics (overall)				
		Length (mm)	Width (mm)	Height (mm)	Volume (mm³)	Total volume (right + left, mm³)
N	Right	17.10±4.89	13.82±4.23	11.67±3.59	1414.37±42.19[a]	2834.32±53.21[a]
	Left	18.06±5.12	14.01±4.53	11.73±3.91	1533.43±64.48[a]	
T	Right	18.71±2.04	14.59±1.47	11.91±0.92	1700.36±53.35[a,b]	3527.73±57.86[b]
	Left	19.69±4.23	14.80±3.21	11.99±0.20	1827.37±72.12[b]	

Testes from twelve N (*n*=8) and T (*n*=4) privately-owned clinically healthy domestic tomcats were collected immediately after routine bilateral orchiectomy. Testicular volume (mm³) for each testis was estimated from the formula for an ellipsoid as follows; Testicular volume = length × height × width × (π/6). Values are means ± s.e.m. Different superscripts (a, b) in a column indicate significant differences between testes (right and left) and donor groups (*P*<0.05). N: normozoospermic; T: teratozoospermic; s.e.m.: standard error of mean

Table 2: Sperm quality parameters in N and T domestic cats

Donor group	Sperm quality parameters			
	Concentration (10^6 cells ml $^{-1}$) (total sperm cells recovered*)	Membrane integrity (%)	Motility (%)	Acrosomal integrity (% in live cells)
N	626.92±112.36[a]	75.15±5.09[a]	78.44±5.62[a]	71.48±4.23[a]
T	952.72±184.26[b]	70.87±4.93[a]	71.20±2.36[a]	62.65±3.33[a]

Spermatozoa from twelve N (*n*=8) and T (*n*=4) privately owned clinically healthy domestic tomcats were collected immediately after bilateral epididymectomy. This study used representative epididymal sperm samples belonging to R3 (cauda) from each group (N and T). Values are means±s.e.m. Different superscripts (a, b) in a column indicate significant differences between donor groups (*P*<0.05). *In the present study, the concentration equals the total sperm number because the volume of PBS was the same in all cases (1 ml). N: normozoospermic; T: teratozoospermic; s.e.m.: standard error of mean; PBS: phosphate-buffered saline

when both groups were analyzed (N and T groups), which accounted for more than 93% of the variability in all cases from the seven initial morphometric descriptors (93.59%, 95.41%, and 94.61% for R1, R2, and R3, respectively [N group], and 93.00%, 94.52%, and 93.10% for R1, R2, and R3, respectively [T group]) (**Table 4**). Both PCs (PC1 and PC2) were constant in each epididymal region regardless of the group analyzed and were used to characterize each cell and classify them in the subsequent cluster analysis.

PCR1N1 was positively related to the dimensional parameters A, P, and L, and to the shape parameters EL and EO, but was negatively related to dimensional parameter W and shape parameter R. PCR1N2 was negatively related to shape parameters (except R) and positively related to all dimensional parameters. The corresponding equations of the PCR1N1 (a) and PCR1N2 (b) are expressed as follows:

(a) 0.2762 A + 0.4129 P + 0.4419 L – 0.1438 W + 0.4138 EL + 0.4140 EO – 0.4409 R

(b) 0.5313 A + 0.3238 P + 0.1757 L + 0.6307 W – 0.2947 EL – 0.3091 EO + 0.0397 R.

PCR2N1 was again positively related to the dimensional parameters A, P, and L, and to the shape parameters EL and EO, but was negatively related to dimensional parameter W and shape parameter R. PCR2N2 was negatively related to the shape parameters EL and elongation and positively related to all dimensional parameters and shape parameter R. The corresponding equations of the PCR2N1 (a) and PCR2N2 (b) are expressed as:

(a) 0.3184 A + 0.4156 P + 0.4342 L – 0.1548 W + 0.4107 EL + 0.4046 EO – 0.4252 R

(b) 0.5147 A + 0.2690 P + 0.1148 L + 0.7148 W – 0.2354 EL – 0.2881 EO + 0.0038 R.

PCR3N1 was positively related to A, P, and L dimensional parameters and R shape parameter, but negatively related to the W dimensional parameter, and EL and EO shape parameters. PCR3N2 was positively related to all dimensional parameters and R shape parameter, and negatively related to EL and EO. The corresponding equations of the PCR3N1 (a) and PCR3N2 (b) are expressed as follows:

(a) 0.2755 A + 0.4138 P + 0.4339 L – 0.1843 W – 0.4056 EL – 0.4192 EO + 0.4359 R

(b) 0.5625 A + 0.2964 P + 0.1704 L + 0.6516 W – 0.2736 EL – 0.2572 EO + 0.0290 R.

PCR1T1 was positively related to the dimensional parameters A, P, and L, and to shape parameters EL and EO, but was negatively related to dimensional parameter W and shape parameter R. PCR1T2 was negatively related to the shape parameters EL and EO and positively related to all dimensional parameters and shape parameter R. The corresponding equations of the PCR1T1 (a) and PCR1T2 (b) are expressed as:

(a) 0.2931 A + 0.4162 P + 0.4611 L – 0.0759 W + 0.3993 EL + 0.4079 EO – 0.4432 R

(b) 0.4961 A + 0.3356 P + 0.1692 L + 0.6127 W – 0.3379 EL – 0.3355 EO + 0.1011 R.

PCR2T1 was positively related to A, P, and L dimensional parameters and EL and EO shape parameters, but negatively related to W, and R shape parameter. PCR2T2 was positively related to all dimensional parameters and R shape parameter, and negatively related to EL and EO. The corresponding equations of the PCR2T1 (a) and PCR2T2 (b) are expressed as follows:

(a) 0.2881 A + 0.4158 P + 0.4462 L – 0.1200 W + 0.4142 El + 0.4103 EO – 0.4363 R

(b) 0.5240 A + 0.3099 P + 0.1536 L + 0.6528 W – 0.2884 EL – 0.3045 EO + 0.0588 R.

Finally, PCR3T1 had similar characteristics to PCR2N1, but PCR3T2 was negatively related to all dimensional parameters and shape parameter R, and positively related to EL and EO shape parameters. The corresponding equations of the PCR3T1 (a) and PCR3T2 (b) are expressed as:

(a) 0.2400 A + 0.3965 P + 0.4499 L – 0.1268 W + 0.4250 EL + 0.4276 EO – 0.4506 R

(b) −0.5265 A – 0.3718 P – 0.2302 L – 0.5814 W + 0.3027 EL + 0.3024 EO – 0.1014 R.

Influence of normozoospermic and teratozoospermic donors on sperm subpopulations after PCA and clustering analysis in the feline species
After the PCA and cluster analysis for R1, R2, and R3, four sperm subpopulations were identified in N and T groups in a data matrix of 18 334 and 12 568 elements. A representation of different model cluster distribution of sperm heads in different donor groups

Table 3: Sperm head morphometric dimensional and shape parameters in different epididymal regions (R1, R2, and R3) in N and T domestic cats

Donor group	Region	Sperm head morphometric parameters						
		Area (μm²)	Perimeter (μm)	Length (μm)	Width (μm)	Ellipticity (–)	Elongation (–)	Rugosity (–)
N	R1	8.42±0.01[a]	11.30±0.01[a]	4.20±0.00[a]	2.27±0.00[a]	1.89±0.00[a]	0.29±0.00[a]	0.83±0.00[a]
	R2	8.22±0.01[b]	11.26±0.01[b]	4.25±0.00[b]	2.19±0.00[b]	1.96±0.00[b]	0.31±0.00[b]	0.82±0.00[a,b]
	R3	8.20±0.01[b]	11.32±0.02[a,c]	4.24±0.01[b]	2.17±0.00[c]	2.00±0.00[c]	0.31±0.00[b]	0.81±0.00[b]
T	R1	8.77±0.02[c]	11.40±0.02[d]	4.18±0.00[c]	2.36±0.00[d]	1.80±0.00[d]	0.27±0.00[c]	0.85±0.00[c]
	R2	8.50±0.02[d]	11.40±0.02[d]	4.22±0.01[d]	2.26±0.00[a]	1.90±0.00[a]	0.29±0.00[a]	0.82±0.00[a,b]
	R3	8.10±0.02[e]	10.96±0.00[e]	4.02±0.01[e]	2.24±0.00[e]	1.82±0.00[d]	0.28±0.00[a,c]	0.84±0.00[a,c]

Values are means ± s.e.m. (6010, 6745 and 5579 spermatozoa [N group] for R1, R2, and R3, respectively; 4053, 3637, and 4878 spermatozoa [T group] for R1, R2, and R3, respectively). Different superscripts (a–e) in a column indicate significant differences among epididymal regions and donor groups (P<0.01). R1: region 1 (caput); R2: region 2 (corpus); R3: region 3 (cauda). N: normozoospermic; T: teratozoospermic; s.e.m.: standard error of mean

according to the subpopulation (CL1 to CL4) is shown in **Figure 1**. The disclosed subpopulations were characterized by different proportions of the sperm head cluster distribution (P < 0.001). Morphometric characteristics of those subpopulations and their distribution in N group and T group are shown in **Tables 5** and **6**, respectively. The frequency of sperm distribution (percentage) within each cluster, as defined after the clustering analysis for different epididymal regions and donor group, is represented in **Figure 2**.

DISCUSSION

In mammals, the phenomenon of teratozoospermia contributes considerably to overall poor reproductive capacity owing to structurally abnormal spermatozoa and reduced oocyte penetration rates.[24,25] The present study demonstrates evident alterations in testicular volume (partial and total) and sperm quality (concentration) in the cauda epididymidis (R3), revealing important differences between teratozoospermic and normozoospermic feline donors. In contrast to the present study, Müller et al.[6] found no differences in testicular size or sperm production between the two donor groups. Our data demonstrated significant differences in sperm morphometric values in different epididymal regions and between donor groups. Marked differences have been found in sperm morphometric subpopulations among epididymal regions and N and T donor groups, and this fact might be associated with fertility, since an unequivocal influence of sperm morphology on feline sperm fertilizing potential has been demonstrated.[26] To our knowledge, this is the first research that has documented relevant findings related to testicular morphometry and sperm subpopulations differences during sperm migration through the epididymal duct in both normozoospermic and teratozoospermic domestic cats.

In feline species, teratozoospermia occurs as a phenomenon induced by several factors including age, season, diet, toxic agents, and disease.[27] However, the existence of teratozoospermic individuals has been associated as well with deleterious effects of high inbreeding that has been related to the loss of genetic variability.[27] These data provide the impetus for future studies of teratozoospermic individuals as an avenue for the determination of inbreeding degree and genetic assessment of domestic and wild felids. As expected in teratozoospermic donors, there was a high percentage of sperm abnormalities, which was confirmed by the objective morphometric analyses carried out throughout the epididymal duct. In the present study, the combination of CASA-Morph with PCA and clustering analyses indicated several morphometric patterns during the sperm maturation process. Although this combination has proven to be efficient, objective and useful for the evaluation of sperm subpopulations in several species, there are no reports that describe the subpopulational characteristics of feline sperm as determined by a CASA-Morph system. Our hypothesis

Table 4: Results from the PC analysis performed on the computerized morphometric analysis of spermatozoa from different epididymal regions (R1, R2, and R3) from N and T domestic cats

Donor group	PC	Initial eigenvalues		
		eigenvalue	Percentage variance explained	Cumulative (%)
N	R1			
	PCR1N1	4.356	62.236	62.236
	PCR1N2	2.194	31.355	93.591
	R2			
	PCR2N1	5.007	71.536	71.536
	PCR2N2	1.671	23.883	95.419
	R3			
	PCR3N1	4.737	67.675	67.675
	PCR3N2	1.886	26.944	94.619
T	R1			
	PCR1T1	4.007	57.246	57.246
	PCR1T2	2.502	35.755	93.001
	R2			
	PCR2T1	4.491	64.170	64.170
	PCR2T2	2.124	30.351	94.521
	R3			
	PCR3T1	3.842	54.893	54.893
	PCR3T2	2.674	38.210	93.104

Initial eigenvalues of the two PCs are given for different donor groups. Percentage of variance is the proportion of the total variance explained by each PC. PCR1N: principal component region 1 normozoospermic; PCR2N: principal component region 2 normozoospermic; PCR3N: principal component region 3 normozoospermic; PCR1T: principal component region 1 teratozoospermic; PCR2T: principal component region 2 teratozoospermic; PCR3T: principal component region 3 teratozoospermic; PCs: principal components; N: normozoospermic; T: teratozoospermic

was that teratozoospermia is reflected in different stages of sperm maturation in the epididymal duct and would produce alterations in the sperm morphometric subpopulations that would explain the reduced fertility potential.[26] In general, our results indicated that spermatozoa from different epididymal regions such as R1 or R2 could be used as predictors of teratozoospermia in domestic cats because of the morphometric differences reported in the present study between N and T groups. Regarding the sperm content in R3, the N group had a significant lower sperm production than the T group. Therefore, from our results, teratozoospermic individuals would not exhibit low sperm concentration in domestic cats. It is still unknown if the alteration of sperm production and the removal rate of damaged cells are the only factors that defined the sperm concentration in teratozoospermic cats. However, this higher sperm concentration in teratozoospermic individuals could be explained in part by the bigger size of the testis observed in the T group. With regard to sperm
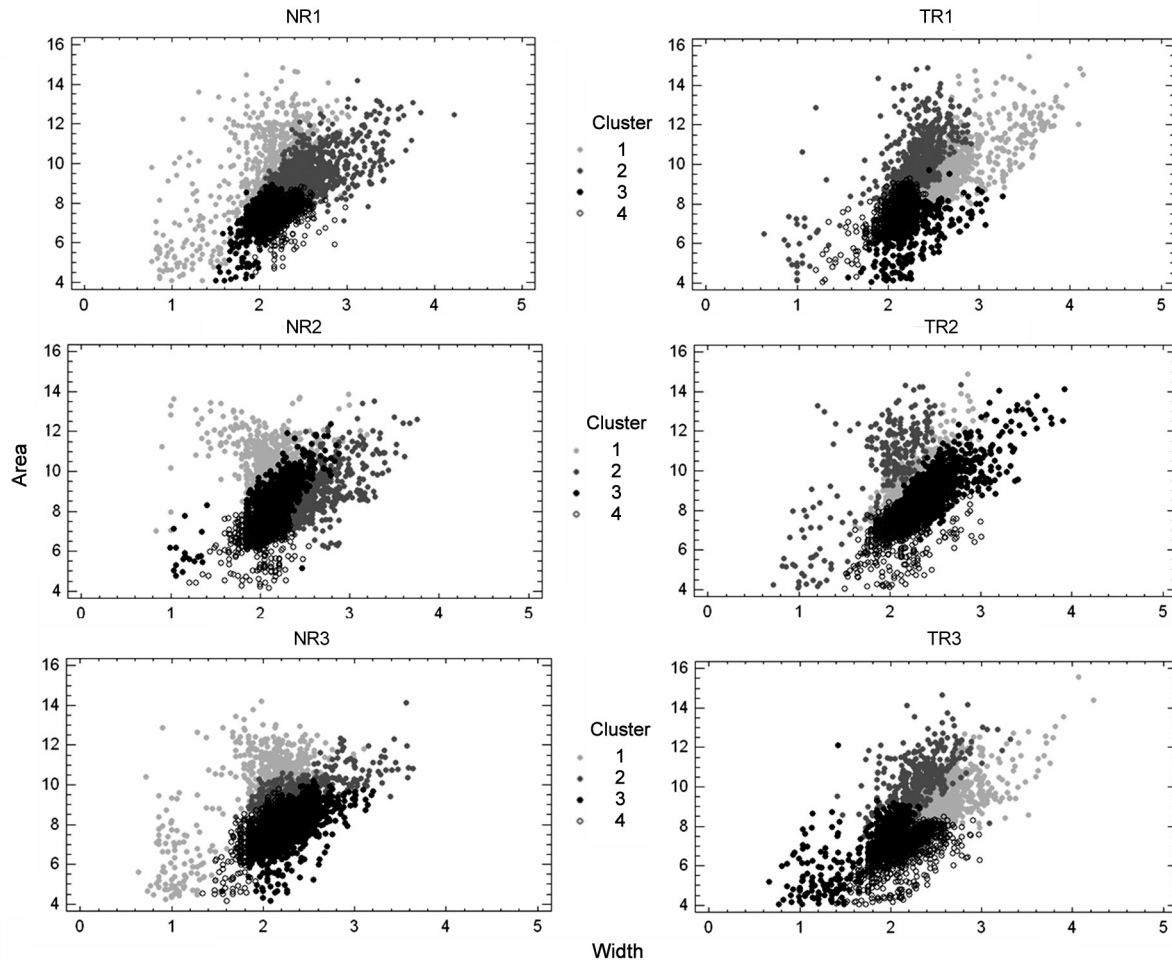
Figure 1: Dot plots of the clusters obtained after the Principal Component and Clustering Analyses derived from the sperm head morphometric data matrix from R1, R2, and R3 epididymal regions in teratozoospermic and normozoospermic domestic cats. Each event represents an individual spermatozoon. Left hand panels, normozoospermic cats (N1, N2, and N3); right hand panels, teratozoospermic cats (T1, T2, and T3). Upper panels, epididymal head (N1, T1); Middle panels, epididymal corpus (N2, T2); Lower panels, epididymal cauda (N3, T3). In each case, there four sperm subpopulations (clusters) were observed (CL1: light gray circles; CL2: dark gray circles; CL3: black circles; CL4: white circles).

Table 5: Distribution of sperm morphometric subpopulations CLs from different epididymal regions (R1, R2, and R3) in N domestic cats

Epididymal region	CL	Number of spermatozoa	Sperm morphometric parameters (centroids)						
			Area (µm²)	Perimeter (µm)	Length (µm)	Width (µm)	Ellipticity (−)	Elongation (−)	Rugosity (−)
R1	CLR1N1	812	9.66	13.59	5.30	2.07	2.68	0.43	0.66
	CLR1N2	2729	8.88	11.42	4.19	2.43	1.74	0.26	0.85
	CLR1N3	1458	7.47	10.52	3.96	2.06	1.92	0.31	0.84
	CLR1N4	1011	7.55	10.26	3.69	2.30	1.60	0.23	0.89
R2	CLR2N1	632	10.42	14.88	6.05	2.08	3.01	0.48	0.60
	CLR2N2	1495	−8.41	10.96	3.94	2.46	1.61	0.23	0.88
	CLR2N3	948	−8.93	12.00	4.62	2.21	2.12	0.35	0.78
	CLR2N4	3670	−7.59	10.57	3.96	2.10	1.88	0.30	0.85
R3	CLR3N1	610	−9.86	14.73	5.87	1.96	3.21	0.49	0.57
	CLR3N2	689	−9.34	12.32	4.67	2.32	2.05	0.33	0.77
	CLR3N3	2643	−7.84	10.59	3.86	2.28	1.69	0.25	0.87
	CLR3N4	1637	−7.67	10.81	4.08	2.03	2.01	0.33	0.82

Values are means for each epididymal region (R1, R2 and R3) and for each subpopulation (CL), respectively. CLR1N (1–4): cluster region 1 normozoospermic. CLR2N (1–4): cluster region 2 normozoospermic; CLR3N (1–3): cluster region 3 normozoospermic; R1: region 1 (caput); R2: region 2 (corpus); R3: region 3 (cauda). n=6010, 6745 and 5579 for R1, R2 and R3, respectively. CLs: clusters; CLR: cluster region; N: normozoospermic

motility, the absence of sperm motility in R1 was expected since it is known that the spermatozoa only acquire motility in R2–R3.[5] Sperm head abnormalities in T group could also explain a higher percentage of sperm membrane damage compared with the N group samples. It

Table 6: Distribution of sperm morphometric subpopulations CLs from different epididymal regions (R1, R2, and R3) in T domestic cats

Epididymal region	CL	Number of spermatozoa	Sperm morphometric parameters (centroids)						
			Area (µm²)	Perimeter (µm)	Length (µm)	Width (µm)	Ellipticity (–)	Elongation (–)	Rugosity (–)
R1	CLR1T1	1662	9.37	11.60	4.17	2.58	1.63	0.23	0.87
	CLR1T2	868	9.97	12.81	4.86	2.31	2.15	0.35	0.77
	CLR1T3	802	7.11	9.97	3.55	2.23	1.60	0.22	0.90
	CLR1T4	721	7.82	10.85	4.08	2.08	1.97	0.32	0.83
R2	CLR2T1	695	9.20	12.21	4.65	2.22	2.10	0.35	0.77
	CLR2T2	249	10.15	14.93	5.99	1.92	3.23	0.51	0.57
	CLR2T3	1792	8.76	11.27	4.10	2.41	1.71	0.26	0.86
	CLR2T4	901	7.00	10.06	3.65	2.08	1.77	0.27	0.86
R3	CLR3T1	2309	8.70	11.23	4.07	2.40	1.70	0.25	0.86
	CLR3T2	402	10.30	13.51	5.14	2.30	2.26	0.37	0.71
	CLR3T3	538	7.24	10.92	4.16	1.82	2.38	0.39	0.76
	CLR3T4	1629	6.97	9.95	3.58	2.14	1.68	0.25	0.88

Values are means for each epididymal region (R1, R2, and R3) and for each subpopulation (CL), respectively. CLR1T (1–4): cluster region 1 teratozoospermic; CLR2T (1–4): cluster region 2 teratozoospermic; CLR3T (1–3): cluster region 3 teratozoospermic; R1: region 1 (caput); R2: region 2 (corpus); R3: region 3 (cauda). n=4053, 3637 and 4878 for R1, R2, and R3, respectively. CLs: clusters; CLR: cluster region; T: teratozoospermic
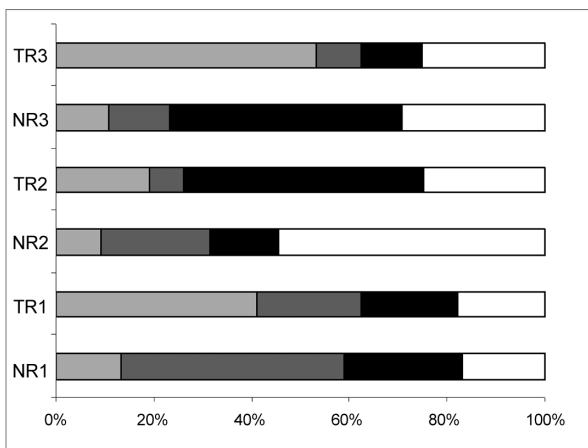

Figure 2: Frequency of distribution (sperm percentage) within each subpopulation, as defined after the Clustering and Discriminant Analyses for different epididymal regions (R1, R2 and R3) and donor groups (N and T). Pattern codes (from left to right) for each subpopulation (cluster) are: light gray for CLR1; dark gray for CLR2; black for CLR3; white for CLR4. Significant differences in the percentage of spermatozoa within each subpopulation for the different donor groups (N and T) were found ($P < 0.001$). NR1: normozoospermic Region 1; NR2: normozoospermic Region 2; NR3: normozoospermic Region 3; TR1: teratozoospermic Region 1; TR2: teratozoospermic Region 2; TR3: teratozoospermic Region 3.

was also observed that the T group contained in a higher percentage of acrosomal damage than the N group, which may affect sperm fertility.[28] Thus, owing to the lack of sperm motility in R1/R2, there is an optimum epididymal duct region (R3) from which to recover spermatozoa in domestic cats for obtaining the best sperm quality, i.e., concentration, motility, viability, and acrosome integrity, simultaneously. The results of the present research are consistent with those of previous studies showing similar sperm quality values for different sperm parameters after epididymal sperm recovery.[5]

It has been described that normal sperm heads present a homogeneous nucleus, which could explain why sperm samples from N group were more homogeneous during epididymal maturation despite having the same number of sperm morphometric subpopulations.[29,30] Thus, this study suggests that sperm subpopulations with different patterns exist in both N and T groups and that they can be successfully

identified by using PCA and multivariate clustering analyses. Our study shows evidence that sperm subpopulations depend on the donor group and the epididymal region analyzed. This may cause changes in sperm traits since the teratozoospermic group revealed differences in sperm head volume (heterogeneous) in the whole population. Our hypothesis was that different results in testicular morphometry and sperm subpopulational structure could be influenced by the teratozoospermia. Using CASA-Morph combined with PCA and clustering analysis, we have demonstrated that there is an association of teratozoospermia with the sperm subpopulation variability. In our study, we obtained four sperm subpopulations in the T group and four in the N group sperm samples. However, there were evident differences in the structure and distribution of these subpopulations, which means that there is an inherent factor related to epididymal sperm maturation, possibly due to physiological differences related to male genetics during the spermatogenic and epididymal maturational processes.[31] In a previous study, our group found that sperm morphometric dimensions and shape parameters could be altered by sperm membrane damage.[20] The present study shows that in the T group, CLR1T3, CLR2T4, and CLR3T4 had the lowest dimensional parameter values, and this fact could be related to defective spermatogenesis. Thus, we could say that these subpopulations may reflect the presence of spermatozoa that could be involved in apoptosis or spontaneously lose their acrosome. The N group had a similar sperm subpopulational structure of the T group, with CLR1N3, CLR2N4, and CLR3N4 being the subpopulations containing the smallest sperm dimensions. However, in the N group, a small size of these subpopulations was not found. It has been described that the membrane fluidity and phospholipid of spermatozoa derived from normozoospermic donors can provide some kind of resistance to plasma and acrosomal membrane damage.[32] This fact could have a direct influence on sperm membrane characteristics and alter the final subpopulations in both normozoospermic and teratozoospermic donors. Whereas spermatozoa contained in CLR1T3, CLR2T4, and CLR3T4 clusters seemed to be morphologically suboptimal, spermatozoa belonging to CLR1T4, CLR3T3, CLR1N2, CLR2N2, and CLR3N3 could represent the morphologically optimal subpopulations characterized by their average sized sperm. It is possible that the predominance of CLR1N2, CLR2N2, and CLR3N3 average sized sperm cells in the N group could be a consequence of optimal membrane structural or biochemical characteristics in contrast to the T group as

described above. These subpopulations could contain standard sized fertilizing spermatozoa, contrary to CLR1T2, CLR2T2, CLR3T2, CLR1N1, CLR2N1, and CLR3N1 which showed large sized sperm subpopulations that could be recognized as bad quality clusters, and which could be related mostly to macrocephalic and immature ejaculated spermatozoa derived from a rapid, defective or incomplete epididymal maturation.[33]

CONCLUSIONS

Testicular morphometric characteristics and epididymal sperm subpopulations observed in the present research clearly indicates differences between N and T donors. Furthermore, sperm head morphometry could be used as a biophysical marker of sperm maturity in feline species and might reflect the different results seen in teratozoospermic donors during sperm maturation. Finally, the alterations of sperm morphometric characteristics during epididymal maturation process could provide valuable information for the identification and clinical diagnosis of teratozoospermic individuals.

AUTHOR CONTRIBUTIONS

MAG-R and MG-H carried out the experiments including sample collection and preparation. MG-H contributed to the conception and study design, experiments, data analysis, drafting, and critical revision of the manuscript.

COMPETING INTERESTS

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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