Animal Reproduction Science 210 (2019) 106176

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Assessment and preservation of liquid and frozen-thawed Black crested mangabey (*Lophocebus aterrimus*) spermatozoa obtained by transrectal ultrasonic-guided massage of the accessory sex glands and electroejaculation☆

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ARTICLE INFO

Keywords:
Primate
Spermatozoa
Ejaculation
Cryopreservation

ABSTRACT

The Black Crested Mangabey (*Lophocebus aterrimus*) is an African monkey listed as Near Threatened by the IUCN and in captivity the population is limited to 34 males. The aim of this study was to evaluate two Black Crested Mangabey males, maintained in captivity in a zoological garden and suspected of infertility, with a complete examination of their genital tract using ultrasonography, followed by recovery of semen using transrectal ultrasonic massage of the accessory sexual glands (TUMASG) and electroejaculation. One male had small testicular and accessory sex gland sizes indicative of senile hypoplasia. The other male was suspected of infertility. Four semen samples were obtained. Fresh semen was initially evaluated, diluted in Refrigeration Medium Test Yolk buffer, cooled at 15 °C and cryopreserved. Endocrine profiles (testosterone, oestradiol, FSH, LH, cortisol), prostatic specific antigen and semen variables (volume, concentration, motility by CASA, viability and acrosome status using flow cytometry, morphology, morphometry utilising TEM) were evaluated in raw, cooled and cryopreserved samples. There was no detrimental effect of cooling or cryopreservation on sperm viability and acrosomal integrity. Similar percentages of viable and acrosome-intact spermatozoa were present in cooled (for 6 h) and frozen-thawed semen samples (75.1% compared with 69.0%, $P > 0.05$), while progressive motility was greater in cooled, compared with frozen-thawed samples (81.5% compared with 67.3%). This study was the first in which there was evaluation of sperm variables in this species and, although this study is limited by the number of animals it provides background information for further studies using assisted reproductive technologies.

☆ Preliminary results have been presented as abstracts in 22nd Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR), Cordoba (Spain), 27–29 September 2018.

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https://doi.org/10.1016/j.anireprosci.2019.106176
Received 16 November 2018; Received in revised form 19 August 2019; Accepted 4 September 2019
Available online 05 September 2019
0378-4320/

1. Introduction

Mangabeys are a group of large African monkeys characterized by moderately projecting snouts, large incisors, hollow cheek bones, long limbs, and long tails. Mangabeys inhabit much of sub-Saharan Africa from Senegal on the west to Kenya and Tanzania on the east (Fleagle and McGraw, 1999; Groves, 2007). The Black Crested Mangabey (*Lophocebus aterrimus*) is an African monkey listed as Near Threatened by the IUCN (Hart et al., 2008), and its population is decreasing in the area south of the Congo River due to habitat loss and human hunting pressure. The major threats are uncontrolled hunting for its meat, and the loss of forest habitat. The captive population in Europe is small, and a European Endangered Species Programme (EEP) was developed to maintain the *ex-situ* population, increase breeding rates, maintain genetic variability and facilitate the study of this species. According to the last report of the Black Crested Mangabey international studbook, there are 82 living captive animals (34 males and 48 females) worldwide in 27 different institutions (International-Studbook, 2018). Life expectancy in the wild for this species has been reported as 32.7 years (Nowak et al., 1999) and, according to the studbook, older recorded ages for zoo-housed animals were 37.1 for males and 36 years for females (Abelló et al., 2018). The youngest sires that reproduced were approximately 4 years old, and oldest sires to have reproduced were approximately 30 to 31 years old.

Scientific knowledge about this species is limited and is focused on karyotype characteristics (Stanyon et al., 1983), transmission of immunodeficiency virus (Takemura et al., 2005) or specific clinical reports (Levecke et al., 2007; Goodall et al., 2018). No information is available about the endocrine values for this species. In relation to the reproductive characteristics, information is very limited. There are reports on the characterization of the menstrual cycle in three female Black Crested Mangabeys and semen recovery using electroejaculation in an 11-year-old male with a lack of reproductive success after 25 artificial inseminations of three females (Calle et al., 1990). To the best of our knowledge, there are no more references about sperm characteristics in this species and none about sperm cryopreservation.

The use of reproductive biotechnologies, particularly cryopreservation of gametes, might provide for a sustainable gene resource for Mangabeys. The storage of gametes is a prerequisite for methods of assisted reproduction (Watson and Holt, 2001; Leibo and Songsasen, 2002; Pukazhenthi and Wildt, 2004), but in all wild species, the first difficulty is related to accessibility of sufficient numbers of sperm cells to be used in assisted reproductive techniques. The most appropriate technique for semen collection should be chosen depending on the physiology, anatomy and behaviour of the target species as well as on the specific circumstances and the individual(s) involved (Holt and Pickard, 1999; Comizzoli et al., 2012; Prieto et al., 2014).

The aim of the present study was to evaluate two Black Crested Mangabey males, maintained in captivity in a zoological garden and suspected of infertility, with a complete examination of their genital tract using ultrasonography, followed by recovery of semen utilising transrectal ultrasonic massage of the accessory sexual glands (TUMASG) and electroejaculation (Santiago-Moreno et al., 2013). After the sperm quality of the fresh semen sample was evaluated, sperm variables were analysed to report the initial descriptive data for these variables in this species. Furthermore, the quality of sperm was evaluated after preservation at refrigeration temperatures and after freezing-thawing.

Additionally, the quantification of hormone concentrations (testosterone, estradiol, FSH, LH and cortisol) and prostatic specific antigen (PSA) in blood samples of these males and two additional males, that served as controls for mature fertile and juvenile males, will provide information for understanding reproduction in this species.

2. Materials and methods

2.1. Animals

The Black Crested Mangabeys were housed in an outdoor exhibit at the Zoo Río Safari Elche (Elche, Spain; 38.2177 °N, 0.6021 °W) where there was natural photoperiod and temperature conditions. One of the males (Pollux, 26 yr, 10 kg) was transferred in 2014 to the reproductive group under the auspices of the EEP program for this species. Pollux had been fertile previously. He, however, was suspected of infertility because after 3 yr in contact with a female (8 yr old) that had menstrual cycling and there were observations of copulation, there was no pregnancy resulting. The older male (Crispin, 36 yr, 11 kg) is a mature male that was isolated from females at the time of study.

Animals were managed during the study in ways that are consistent with the Spanish Policy for Animal Protection (RD 53/2013), which conforms to European Union Directive 2010/63/EU regarding the protection of animals used in scientific experiments. This project was positively evaluated by the Ethical Committee of Animal Experimentation (CEEA 517/2018) and Biosecurity Committee (CBE 183/2018) at the University of Murcia. Also Dr. Tjerk ter Meulen, as European stud bookkeeper (ESB) for *Lophocebus aterrimus*, was informed and provided approval for conducting the study.

Two blood plasma samples were evaluated from two additional fertile males provided by Dr. Tjerk ter Meulen, as European stud bookkeeper (ESB) for *Lophocebus aterrimus.* Joachim, was a male sampled when he was 17 yr of age. He had previously mated with females with the result being production of six offspring in total and one of these was born 8 months after collection of the blood samples. He was a model mature and fertile male. Blood was collected from the other additional male, Wladek, when he was 2 yr of age. Wladek mated with females later in life and these matings resulted in production of six offspring in total and the first offspring was produced when he was 6 years old. He, therefore, serves as a model juvenile male, that became fertile as he matured. Collected blood samples were stored at -20 °C and were transferred from the Netherlands to the University of Murcia for hormonal profile evaluation.

2.2. Management and anaesthesia of the animals

Food and water were withheld for 24 h before the males were anaesthetized using a projectile dart providing intramuscular ketamine hydrochloride (5.5 mg/kg body weight, Anesketin, 100 mg/ml, Dechra Veterinary Products SLU, Barcelona, Spain) plus medetomidine (0.07 mg/kg body weight, Sedastart, 1 mg/ml, B. Braun Vetcare SA, Rubí, Barcelona, Spain). After induction of anesthesia, the animals were moved to the surgical room, where they were monitored by using a pulse oximeter measuring heart rate and percentage of haemoglobin in the blood that is saturated with oxygen (SpO_2, %). There was maintenance of heart rate and oxygen saturation at normal values (65–75 beats/min and 95–100% SpO_2) throughout the experimental period (60–90 min).

2.3. Genital tract evaluation and semen recovery

Ultrasonic evaluation of the genital tract and semen collection were performed on 8 February 2018 for Pollux and Crispin. Testicular size was recorded by measuring the scrotal circumference at its widest diameter with an orchidometer. After clipping the hair around the penis and cleaning the surrounding area, the penis was manually extruded; it was maintained in a protruded state by holding it with the use of gauze just caudal to the glans. The penis was then cleaned with a sterile gauze wetted in a sperm-washing solution composed of Tris, citric acid, and glucose (345 mOsm, pH 6.8). Ultrasonic examination of the prostate, seminal vesicles, and ampulla of the vas deferens was performed using real-time transrectal ultrasonography with a 7.5 MHz linear array probe (Prosound 2, Aloka CO., LTD, Tokyo, 181–8622 Japan). The TUMASG was performed using a ultrasonographic probe placed on the seminal vesicles and the ampulla of the vas deferens, with performing of a back-and-forth motion to induce the expulsion of the spermatozoa (Ungerfeld et al., 2015). This massage was alternated with manual massage of the penile, perineal and pelvic areas of the urethra to transport the ejaculatory fluid through the urethra into the collection tube (graduated glass collection vessel designed for this purpose). The procedure was repeated several times, during a period of approximately 10 min. If the animal did not ejaculate, electrical stimuli (2–5 V lasting 5 s) were provided with an electroejaculator (P–T Electronics, PTE Model 304, Oregon, USA) with intermittent cessations for TUMASG. Prior to insertion into the rectum, the electroejaculator probe was coated with carboxymethyl cellulose gel (Eko Gel®, Papeles Registrales S.A., Barberá del Vallés, Barcelona, Spain) to improve electrical conductivity. The process was monitored using ultrasonic scanning of the ampulla of the vas deferens, verifying that the emptying of the glands was complete. Stimulation was halted when the echogenicity of the ampulla indicated this structure did not contain any more seminal fluid. After collecting the semen, animals received an injection of meloxicam (anti-inflammatory and analgesic NSAID; 0.2 mg/kg body weight s.c., Loxicom 5 mg/ml, Karizoo, Caldes de Montbui, Barcelona, Spain), selenium (1.15 mg/kg body weight) and vitamin E (3.75 U/kg body weight) i.m. (Selevit Complex, Syva, San Andrés del Rabanedo, León, Spain). The anaesthesia was reversed with the intramuscular administration of atipemazole (4 mg Sedastop, 5 mg/ml, B. Braun Vetcare SA, Rubí, Barcelona, Spain). Animals recovered fully within 20–40 min.

2.4. Hormonal and total Prostatic Specific Antigen (PSA) measurements in blood plasma

To evaluate hormone concentrations, blood samples were obtained during anaesthesia in lithium heparin collection tubes (BD Vacutainer®, Becton Dickinson Co., Plymouth, UK) and centrifuged at 1500 *g* for 15 min. The plasma was immediately separated and stored at −20 °C until hormone quantifications were conducted.

Plasma testosterone concentrations were quantified using a radioimmunoassay in duplicate plasma aliquots (100 µL) as previously described (Santiago-Moreno et al., 2005). The sensitivity of the assay was 0.05 ng/mL.

Cortisol concentrations (ng/mL) were quantified using a commercial ELISA kit (Cortisol ELISA Demeditec Diagnostics GmbH (Kiel, Germany). The sensitivity of the assay was 3.79 ng/ml and the intra-assay CV was 6,8% ± 0,9 (n = 20).

Estradiol, FSH, LH and total PSA were quantified using an electrochemiluminescence immunoassay (Elecsys® Estradiol III, FSH, LH and total PSA Assays), using a Cobas e 411 analyzer (Roche Diagnostics, Barcelona, Spain).

2.5. Sperm preservation and cryopreservation

After 30 min of liquefaction, raw semen samples were evaluated (volume, sperm concentration, motility and viability). The liquid fraction from semen samples 1, 2 and 3 were mixed and diluted 1:1 in Refrigeration Medium Test Yolk buffer with Gentamicin Sulphate (Irvine Scientific. Santa Ana, CA, USA) and cooled at 15 °C (Cold sample) (Lange and Bormann, 2017). (Fig. 1).

Refrigerated samples were evaluated after 0.5 h at the zoo laboratory and then the samples were transported from the zoo to the University of Murcia laboratory in a controlled temperature box (15 °C) and evaluated after 6, 24 and 30 h.

Semen Sample 4 was cryopreserved by the dropwise addition of Freezing media (Test Yolk buffer with Glycerol & Gentamicin, Irvine Scientific. Santa Ana, CA, USA) to the liquid fraction until a final 1:1 (v/v) dilution was achieved (Nallella et al., 2004). Cryotubes with the mixture were maintained to equilibrate for 10 min at room temperature (20 C) and later exposed to liquid nitrogen vapour for 10 min before storing in a liquid nitrogen tank. For thawing, the cryotube was maintained for 10 min at room temperature and for 10 additional minutes at 37 °C before evaluation of the frozen-thawed sample.

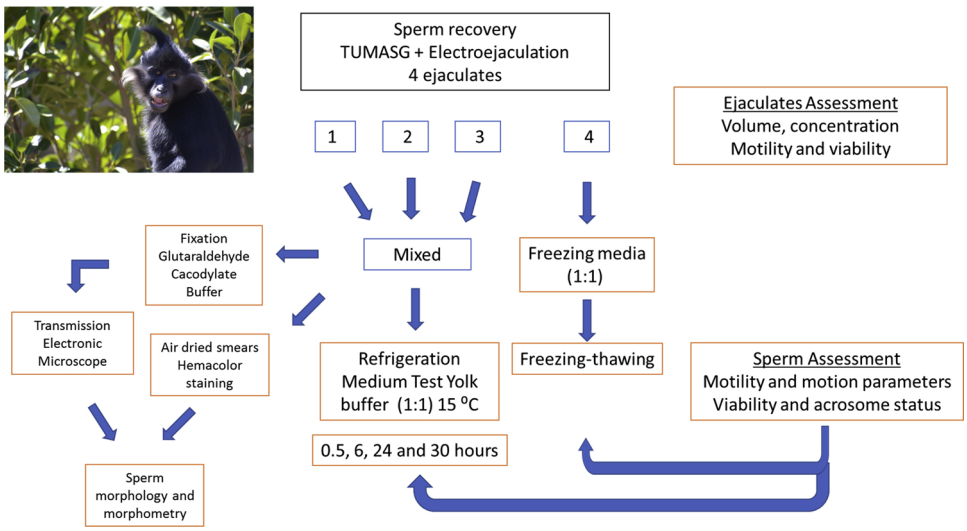

Fig. 1. Experimental design: Sperm recovery from Crispin by TUMASG and electroejaculation. Four ejaculates were analysed for volume, sperm concentration, motility and viability. Sperm morphology and morphometry were evaluated by hemacolor staining and transmision electronic microscope. Ejaculates were refrigerated for as long as 30 h or frozen-thawed. Sperm assesstment of motility, viability and acrosome were conducted using CASA and flow cytometry.

2.6. Sperm assessment

2.6.1. Motility variable assessments using CASA

A 4-μL drop of the semen sample was placed on a warmed (37 ˚C) motility chamber (Spermtrack, Proiser R + D, Valencia, Spain). Five different fields from each sperm sample were evaluated at 100x magnification in a negative contrast phase microscope-attached CASA system (ISAS v1, Proiser R + D, Valencia, Spain). The following CASA-derived motility characteristics were analyzed: total motility (%), progressive motility (%), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity of the curvilinear trajectory (LIN, ratio of VSL/VCL, %), straightness (STR, ratio of VSL/VAP, %), wobble of the curvilinear trajectory (WOB, ratio of VAP/VCL, %), amplitude of lateral head displacement (ALH, μm) and beat cross-frequency (BCF, Hz). Calibrations for the measurements were the same as for human samples (Gadea et al., 2011). Evaluations were conducted 6 h after initiation of the preservation period at 15 ˚C and in frozen-thawed samples.

To investigate the data in more detail, there was use of a cluster analysis, which was conducted to classify the entire set of individual spermatozoa into functionally meaningful subsets, so that the data can be interpreted more easily and more accurately (Holt et al., 2007).

2.6.2. Assessment of viability and sperm acrosomal integrity

Viability and acrosomal status were simultaneously assessed by staining with propidium iodide and the lectin PNA-FITC. Fluorescence was quantified using a flow cytometer (Guava Easycyte 6-2 L, Millipore, Hayward, CA, USA) with a 488 nm (blue) laser, FL-1 sensor, a 525/30 nm band-pass filter to detect FITC-PNA, and a FL-2 sensor and a 695/50 nm band-pass filter to detect PI. Proportions of live acrosome intact, live acrosome damaged and dead spermatozoa were recorded (Gadea et al., 2013). Evaluations of refrigerated samples were conducted after 0.5, 6, 24 and 30 h of preservation at 15 ˚C and in frozen-thawed samples.

2.6.3. Sperm morphology and head morphometric analysis

Semen smears were air-dried and stained using Hemacolor® (Merck KGaA, Darmstadt, Germany). After staining was completed, the entire slide was permanently sealed using Eukitt® mounting medium (Panreac Quimica S.L.U., Barcelona, Spain) and a coverslip.

Sperm morphoabnomalies were evaluated in 200 cells using a microscope (200x magnification) and classified. Sperm head morphometric analysis was performed by computer-assisted sperm analysis system (CASA-Morph) using Sperm-Class Analyzer® v.5.3.0.1 software (Microptic SL, Barcelona, Spain). Sperm head length, width, area and perimeter data were determined for 100 cells of each sample (Esteso et al., 2015).

2.6.4. Transmission electron microscopy

Spermatozoa were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and incubated at 4 ˚C for 2 h. After fixation, spermatozoa were post-fixed in potassium ferrocyanide-reduced osmium tetroxide for 1 h. After extensive washing, the samples were then dehydrated in a graded series of increasing concentrations of ethanol solutions and processed for embedding in Epon 812 (Sigma-Aldricht, Madrid, Spain). Ultrathin sections were obtained using an ultramicrotome (Microm International GmbH, Walldorf, Germany) and mounted on Formvar®-coated nickel grids (Agar Scientific Ltd., Stansted, Essex UK). Ultrathin sections were

counterstained with uranyl acetate followed by use of lead citrate and imaged utilising a Jeol JEL-1011 Transmission Electron Microscope (Jeol, Tokyo, Japan). ImageJ (1.52a) software (Schneider et al., 2012) was used for morphometric evaluation of the ultrastructural images. Length, width, area and perimeter data from sperm nuclei, intermediate piece, principal piece and mitochondria were recorded.

2.7. Statistical analysis

Data are provided as means ± SD. ANOVA was applied to explore the cytometric and CASA seminal variables in refrigerated compared with frozen-thawed samples. When a significant effect of the treatment was detected, the Tukey's *post hoc* test was conducted ($P < 0.05$).

Cluster analysis was used to evaluate the sperm subpopulations based on motion variables as previously described (Holt et al., 2007; Martinez-Soto et al., 2011). The initial data set consisted of 182 cases that contained data values for VAP, VSL, BCF and ALH. After cluster analysis, the data were separated into three groups, summarized in Table 7. In Group 1 there was a slow non-linear transiting of spermatozoa, in Group 2 medium velocity linear transiting spermatozoa and in Group 3 very rapidly transiting of motile cells and linear transiting sperm populations.

2.8. Experimental design

Ultrasonic evaluations of the genital tract were performed for Pollux and Crispin. Spermatozoa were collected from only one male (Pollux). Four semen samples were obtained in ejaculates successively collected (Fig. 1). The liquid fraction from seminal samples 1, 2 and 3 were refrigerated for as long as 30 h and seminal sample 4 was frozen. Sperm assessment was conducted immediately after ejaculate recovery, during the refrigeration period (0.5, 6, 24 and 30 h) and after thawing the frozen samples.

For hormonal and total Prostatic Specific Antigen (PSA) quantifications in blood plasma, there was use of plasma from four animals, two samples collected from Pollux and Crispin on the same day as ultrasonic evaluations and two additional, previously collected samples stored by Dr. Tjerk ter Meulen, as European stud bookkeeper (ESB) for *Lophocebus aterrimus*.

3. Results

Data for ultrasonic genital tract evaluations are included in Table 1. Data for hormonal and total Prostatic Specific Antigen (PSA) quantifications in blood plasma are shown in Table 2. Values for FSH and LH were less than the minimum detection concentration (< 0.1 mUI/mL) in all the samples.

Spermatozoa were collected from only one male (Pollux). In addition to TUMASG, four repetitions of electroejaculation (six electrical stimuli per cycle) were required to induce ejaculation. A total of four semen samples were collected during a period of 90 min, three of the samples were composed of a coagulum and a liquid fraction (Table 3).

Data for semen variables (volume of liquid fraction, sperm concentration, total motility and viability) from every raw sample are shown in Table 3. Except for sample no. 1 in which there was a relatively lesser sperm concentration than in the other samples, sperm motility and viability were greater than 60% (Table 3).

Values for morphometric variables of sperm measured using CASA-Morph and TEM are shown in Table 4 and are depicted in Figs. 2 and 3. In relation to sperm morphological abnormalities, 42% of the spermatozoa had some type of morphological alteration, most frequently bent or coiled tails (34%), head damage (3%) or headless (3%), neck damage (1%) and intermediate region alterations (1%) (Fig. 3).

Viability and acrosome status was simultaneously assessed using flow cytometry in refrigerated and frozen-thawed samples. During refrigeration the percentage of viable cells decreased from 92% at to 0.5 to 84% at 6 h (Table 5, $P < 0.05$). After 24 and 30 h of refrigeration, sperm viability decreased to 75% (Table 5, $P < 0.05$). The percentage progressive motility was 81.5% at 6 h (Table 6). At 24 and 30 h, however, the sperm motility was not progressive and only vibrations of the tail were detected; it was therefore not possible to quantify motion variables using CASA.

Frozen-thawed samples, however, had a viability of 68% and 67% progressive motility. Comparing refrigerated (6 h) and frozen-thawed samples, the total sperm viability was greater in refrigerated than frozen-thawed samples (84.01 ± 1.93 compared with 68.54 ± 3.02, $P < 0.05$ Table 5) as was the percentage of viable spermatozoa with acrosome damage (spontaneous or not induced acrosome reacted) (8.93 ± 1.80 compared with 0.51 ± 1.01, $P < 0.05$ Table 5). The percentage of viable spermatozoa with an intact acrosome was similar in both groups (75.08 ± 2.76 compared with 69.03 ± 2.58, $P > 0.05$. Table 5).

Table 1
Ultrasonic genital tract evaluation and plasma testosterone concentrations.

Male	SC	TD	SV	Pr	Amp
Pollux	15.8	1.73	0.55	0.54	0.24
Crispin	6.0	0.92	0.49	0.42	NA

SC: Scrotal circumference (cm). TD: Testicular diameter (cm). SV: Seminal vesicles (area, cm^2) Pr: Prostate (area, cm^2). Amp: Ampullary dilatation (diameter, cm), NA = not applicable.

Table 2
Plasma hormone concentrations and total Prostatic Specific Antigen (PSA).

	Pollux	Crispin	Joachim	Wladek	Mangabey reference
Age[*] (yr)	26	34	17	2	
Testosterone (ng/mL)	2.3	4.6	6	0.4	5.95[†]
Estradiol (ng/mL)	5.4	5.0	12.29	5.0	
FSH (mUI/mL)	< 0.1	< 0.1	< 0.1	< 0.1	
LH (mUI/mL)	< 0.1	< 0.1	< 0.1	< 0.1	1.8 µg/mL[†]
Cortisol (ng/mL)	733,7	425,3			45-58 ng/mL[†]
Total PSA (ng/mL)	4.46	0.04	0.02	0.02	

* Age when blood was sampled.

† Mann, DR, VD Castracane, F McLaughlin, KG Gould, and DC Collins 1983 Developmental patterns of serum luteinizing hormone, gonadal and adrenal steroids in the sooty mangabey (Cercocebus atys); *Biol Reprod* 28.279–284.

Table 3
Semen variables of raw semen samples obtained by transrectal ultrasonic-guided massage of the accessory sex glands (TUMASG).

Semen sample	Macroscopic aspect	Coagulum presence	Volume (liquid fraction, µL)	Sperm concentration (x 10^6/mL)	Viability (%)	Motility (%)
1	Cloudy	Yes	150	0.3	55	30
2	Cloudy	Yes	50	1	80.1	60
3	Yelowish	No	750	1.5	87.3	70
4	Cloudy	Yes	400	3	93	> 80

Table 4
Morphometric variables of spermatozoa measured by transmitted light microscopy and transmission electron microscopy (mean ± SD).

	Length (µm)	Width (µm)	Perimeter (µm)	Area (µm^2)
Sperm head[*]	5.18 ± 0.26	3.80 ± 0.21	15.22 ± 0.75	16.60 ± 1.58
Sperm nuclei	4.07 ± 0.46	0.94 ± 0.15	9.79 ± 1.06	3.16 ± 0.65
Intermediate piece	8.51 ± 0.56	0.96 ± 0.12		
Principal piece	4.54 ± 0.04	0.55 ± 04		
Mitochondria			0.06 ± 0.02	0.86 ± 0.11

* Measeured by CASA-Morph.

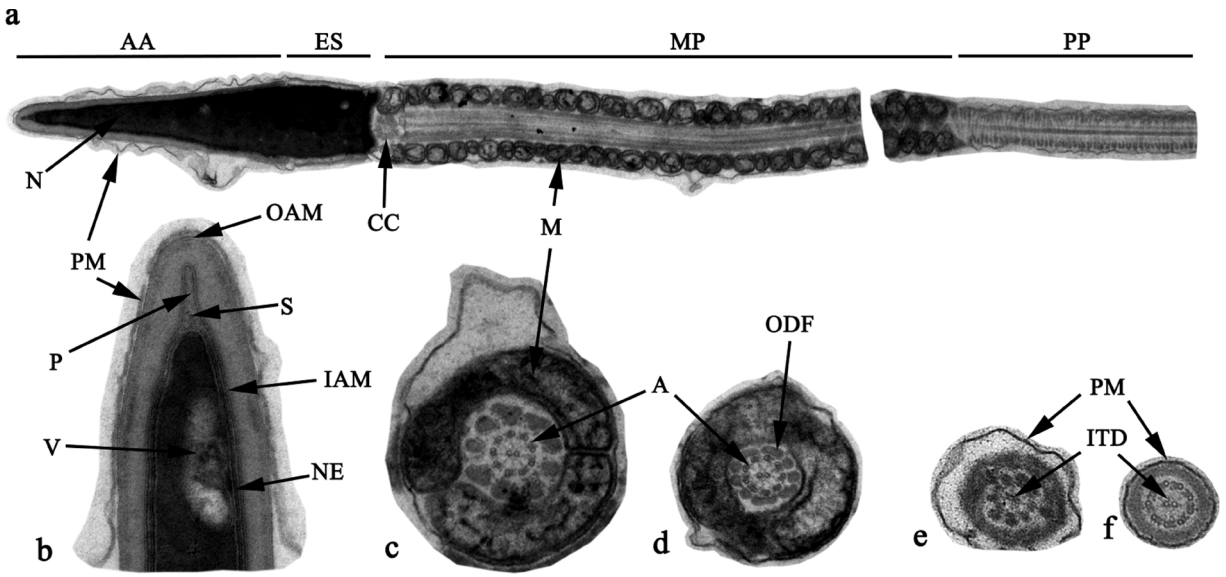

Fig. 2. Ultrastructural analysis of black crested mangabey sperm: (a) Longitudinal section of a sperm, (b) Detail of the anterior acrosome area (AA) of the head, (c)-(f) cross section of sperm tails: (c) proximal midpiece (MP), (d) distal midpiece, (e) principal piece (PP), and (f) end piece. A, axoneme; CC, centriole cylinder; ES, equatorial segment; IAM, inner acrosomal membrane; ITD, inner microtubules doublet; M, mitochondria; N, nucleus; NE, nuclear envelope; OAM, outer acrosome membrane; ODF, outer dense fibers; P, perforatorium; PM, Plasmatic membrane; S, electron-dense material of the subacrosomal space; V, vacuole in the sperm nucleus; N, nucleus.

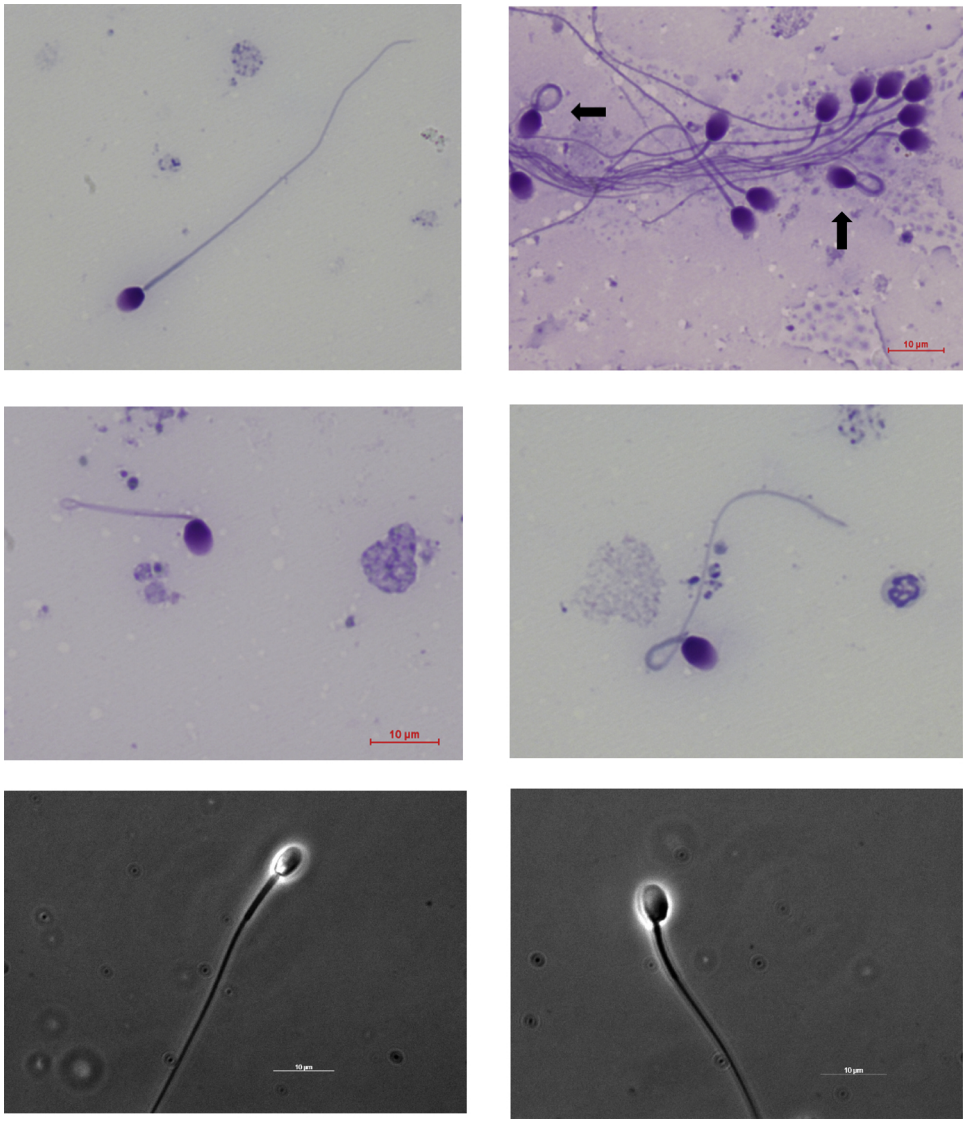

Fig. 3. Microscopic images of Black Crested Mangabey spermatozoa: A–D. Stained by Hemacolor® (Merck KGaA, Darmstadt, Germany) and observed x1000 magnification. A. Normal morphology. B. Normal morphology and swollen tails (black arrow). C. Bent tail. D. Bent tail and intermediate piece alteration. E–F. Contrast phase microscopy. × 1000 magnification. E. Normal morphology. F. Abaxial tail implantation.

Table 5
Viability and acrosome status of refrigerated and frozen-thawed semen samples (Mean ± SD).

Treatment	Viable intact acrosome (%)	Viable damaged acrosome (%)	Non-viable spermatozoa (%)
Refrigerated 0.5 h	83.79 ± 0.40 [a]	8.18 ± 2.65 [a]	8.04 ± 2.38 [a]
Refrigerated 6 h	75.08 ± 2.76 [b]	8.93 ± 1.80 [a]	15.99 ± 1.93 [b]
Refrigerated 24h	60.09 ± 4.43 [c]	15.64 ± 3.45 [b]	24.26 ± 3.56 [c]
Refrigerated 30 h	58.44 ± 3.27 [c]	15.71 ± 2.00 [b]	25.85 ± 3.91 [c]
Frozen-thawed	69.03 ± 2.58 [b]	0.51 ± 1.01 [d]	31.46 ± 3.02 [d]

[a, b, c, d]In the same column represent differences $P < 0.05$.

In relation to sperm motility and motion parameters, sperm in samples refrigerated for 6 h had greater progressive motility, WHO Type A, VCL, ALH and BCF than frozen-thawed samples, and lesser wobble of the curvilinear trajectory (WOB, ratio of VAP/VCL, %) (Table 6, $P < 0.05$). These findings were confirmed when a sperm subpopulation cluster analysis was conducted (Table 7). The proportion of sperm with a great amount of velocity and linear movement (Cluster 3) was greater in refrigerated than frozen-thawed samples (20.59 compared with 11.49%, $P < 0.05$) and there was the opposite finding for lesser velocity spermatozoa (Cluster 1)

Table 6
Motility variables of refrigerated and frozen-thawed samples measured by CASA.

Treatment	Progressive motility (%) (A + B type)	Type A WHO (%)	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	LIN (%)	STR (%)	WOB (%)	ALH (μm)	BCF (Hz)
Refrigerated 6 h (n = 39)	81.45[a]	71.79[a]	84.01[a]	67.41	69.03	73.87	90.80	77.69[a]	2.19[a]	10.07[a]
Frozen-thawed (n = 214)	67.29[b]	51.40[b]	68,86[b]	58,59	60,02	80.99	92.40	84.74[b]	1.71[b]	7.52[b]
P-value	0.02	0.02	0.02	0.23	0.19	0.11	0.58	0.04	< 0.01	< 0.01

CASA, computer-assisted sperm analysis; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity of the curvilinear trajectory; STR, straightness; WOB, Wobble (VAP/VCL); ALH, amplitude of lateral head displacement; BCF, beat cross-frequency.
[a, b]In the same column represent differences $P < 0.05$.

(44.12 compared with 57.43%, $P < 0.05$).

4. Discussion

This is the first report to our knowledge of ultrasonic genital, endocrine, sperm characteristic and preservation of spermatozoa evaluations in the Black Crested Mangabey species. The limitation of this study is the small number of males evaluated ($n = 2$) and only the two additional blood samples from the other males for which there were hormonal and PSA assessments. There, however, are only 34 males of this species in captivity according to the last stud book (International-Studbook, 2018), so the proportion of animals studied from available animals is 5.9%.

Most non-primate animals require electrostimulation under anaesthesia to collect semen (Bader, 1983). Successful electroejaculation has been accomplished in several primate species such as chimpanzees (*Pan troglodytes*), orangutans (*Pongo pygmaeus*), gorillas (*Gorilla gorilla*), baboons (*Papio hamadryas anubis*), Tana Mangabeys (*Cercocebus galeritus*), red-capped Mangabeys (*Cercocebus torquatus torquatus*), etc. (Warner et al., 1974; Kyaligonza, 1998; Morrell and Hodges, 1998; Hernandez-Lopez et al., 2002; Wolf, 2009; Maya-Soriano et al., 2012). The TUMASG approach is an alternative procedure for semen collection in which ultrasonic assessments of the ampulla glands was useful for monitoring the ejaculatory response. In addition, this technique requires very few subsequent electrical electroejaculation stimuli and sometimes none at all. The effectiveness of TUMASG was initially developed for the aoudad (*Ammotragus lervia*) (Santiago-Moreno et al., 2013) and subsequently for the Iberian ibex (*Capra pyrenaica*), and mouflon (*Ovis musimon*) (Ungerfeld et al., 2015). Currently it is a common technique utilised for semen collection in several wild ruminant species (Pradiee et al., 2016).

This is the first study using the TUMASG procedure in primates. Results indicate that four cycles (six electrical stimuli per cycle; total 24 electrical stimuli) with intermittent cessations in stimuli for TUMASG were required to induce ejaculation in the Black Crested Mangabey. Results of the present study indicate that the TUMASG procedure is less effective in primates than in ruminants. Nonetheless, it should be a useful method for minimizing the total number of electrical stimuli needed for semen collection, and for utilising a lesser voltage and duration of electrical stimuli, thus shortening the procedures for semen collection. The risks associated with thermal damage and myopathy should be reduced, therefore, with practical advantages in field conditions for semen collection. In non-human primates, there is generally a need for several series of multiple electrical stimuli for successful electroejaculation. The TUMASG procedure has some advantages with respect to electroejaculation of squirrel monkeys where three series (cycles) composed of 35 electrical stimuli of increasing current (12.5–100 mA) (Oliveira et al., 2015), in great apes where there was need for 12 cycles composed of six rhythmic stimulations (20 mA–170 mA) (Warner et al., 1974) and for chacma baboons (*Papio ursinus*) where six cycles were needed composed of 12 rhythmic stimulations (2.5–4.5 V) (Bornman et al., 1988) for semen collection.

The prostate is located at the distal end of the urinary bladder in the Black Crested Mangabey, as in cynomolgus macaques, rhesus macaques, baboons (*Papio hamadryas anubis*), and common marmosets (*Callithix jacchus*) (Mubiru et al., 2008). Scrotal circumference and the testicular diameter values in Pollux were two time larger than those of the Crispin. Results from ultrasonic assessments and sperm evaluations of Pollux indicate he should be a fertile male, and thus the lack of offspring could be related to fertility problems of the female or other causes. In contrast, Crispin had a small testicular size and accessory sex glands size, indicative of senile hypoplasia, which is the contrasting condition as compared with benign prostatic hyperplasia in aged men (Unnikrishnan et al., 2017). The values with evaluation of total PSA, however, were greater for Pollux (4.46 ng/mL) than the values for the other males (0.02 -0.04 ng/mL) and were similar to the reference limit for humans (4 ng/mL). Crispin, however, had relatively lesser values for PSA than the other males in this study.

Values for FSH and LH were less than the detection concentration (< 0.1 mUI/mL) in all the samples, indicating there is a lack of sensitivity of the antibodies used. While using the same methodology oestradiol concentrations were quantifiable. Values of oestradiol for the males were less than those for the reference mature male (Joachim; 5–5.4 compared with 12.29 pg/mL) and are similar to concentrations in the juvenile male (Wladek, 5.0 pg/mL). In male green monkeys (*Cercopithecus sp*) diurnal values for oestradiol ranged 5–10 pg/mL, followed by a marked increase of oestradiol was recorded during dark hours of the circadian biorhythm (Beattie and Bullock, 1978).

Cortisol concentrations were greater than anticipated (733.7 and 425.3 ng/mL) indicating the management and anaesthesia procedures were stressful for the animals. Other causes for this greater than anticipated concentration of cortisol could be related to health problems (none detected in these animals) or social interactions that affect cortisol concentrations as previously reported in

Table 7
Sperm subpopulations grouped by cluster analysis of motion variables.

Cluster	Group	Proportion	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	LIN (%)	STR (%)	WOB (%)	ALH (μm)	BCF (Hz)
1	Slow non-linear spermatozoa	54.95	46.90	30.88	34.42	68.64	86.92	75.63	1.70	7.07
2	Medium velocity linear spermatozoa	31.87	89.67	82.19	81.69	91.48	98.12	91.50	1.88	9.47
3	Very fast and linear spermatozoa	13.19	131.56	129.45	127.08	97.04	99.14	96.41	1.99	8.34

VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity of the curvilinear trajectory; STR, straightness; WOB, Wobble (VAP/VCL); ALH, amplitude of lateral head displacement; BCF, beat cross-frequency.

faecal samples of wild gray-cheeked mangabeys (*Lophocebus albigena*) (Arlet et al., 2009). Nevertheless, there are no basal reference values for serum/plasma for the Black Crested Mangabey species. Values in male Cynomolgus Monkeys (*Macaca fascicularis*) ranged 10–35 ng/mL with significant changes related to the circadian cycle and individual differences (Czoty et al., 2009) and 48–58 ng/mL in Sooty Mangabey (*Cercocebus atys*) (Mann et al., 1983).

The testosterone concentration in one of the individuals (Pollux, 2.3 ng/ml) was greater than in the juvenile male (Wladek, 0.4 ng/mL) and less than in a fertile mature male (Joachim, 6 ng/mL) and those reported for adult (> 6 yr old) Sooty Mangabeys (*Cercocebus atys*) (5.95 ng/mL) (Mann et al., 1983). Several factors may affect serum testosterone concentrations, such as the hierarchical status, the presence of periovulatory females and the environmental temperature (Arlet et al., 2011). The basal concentrations in Pollux was adequate for development and maintenance of spermatogenic functions. This may be explained because testosterone is a steroid secreted in a pulsatile manner, and thus using only one blood sample to quantity testosterone concentrations can lead to misinterpretations of actual concentrations. Nevertheless, the percentage of sperm with morphological abnormalities was considerable (42%) in Pollux, similar to that when sperm were procured using electroejaculation (46%) and epididymal harvesting (39%) in one male red-capped mangabey *(Cercocebus torquatus torquatus)* (Maya-Soriano et al., 2012) and greater than that reported for Tana Mangabeys (*Cercocebu sgaleritus*) (16%) (Kyaligonza, 1998); thus, an endocrine dysfunction should not be discounted when these comparisons are made with thos for Pollux. Bent and coiled sperm tails were the most common sperm abnormalities. Although these minor defects may be common primary abnormalities (Chenoweth, 2005), these can also be induced by variation in osmolality of the semen sample (Correa-Perez et al., 2003) related to the TUMASG and electroejaculation procedures.

Scanning electron microscopy has been used to evaluate morphology and morphometry of primate spermatozoa (Flechon et al., 1976) and for morphological comparisons among primate species (Martin et al., 1975; Gould and Martin, 1978). Transmission electron microscopy has also been used for evaluation of primate spermatozoa (Bedford, 1967; Zamboni et al., 1971). Transmission electron microscopy is a procedure that can be used for the precise evaluation of sperm morphology (Moretti et al., 2016). To best of our knowledge, this is the first report about sperm morphology, morphometry and ultrastructure of the Black Crested Mangabey species.

Use of the TUMASG technique and electroejaculation led to the production of four ejaculates from one male in a 1-day period. The initial sperm quality in the ejaculates collected in the present study indicated this quality was satisfactory for investigation of effects of preservation procedures on sperm viability. Some problems related to the samples recovered using electroejaculation in primates are the presence of urine in the sample, which is a possibility for sample 3. Sample no. 3 had a yellowish color and spermatozoa observed using microscopy indicated there was swelling of the sperm tails that have been reported to be induced when there are hypoosmotic conditions in the media in which sperm are stored (Jeyendran et al., 1992). All these data indicate the samples were contaminated with urine (Makler et al., 1981; Griggers et al., 2001). The rapid dilution in refrigeration isosmotic media generally results in a hypoosmotic condition that results from urine contamination (Makler et al., 1981). Motility of the sample immediately after dilution in refrigeration media (85%) was slightly greater compared with the motility of the raw samples (range 30%–70%). The presence of persistent coagulum after a normal liquefaction time is a common problem with primate ejaculates (Greer et al., 1968), which could be solved in some cases with trypsin treatment (Roussel and Austin, 1967). Nevertheless, trypsin treatment could induce damage to the spermatozoa, and because of this trypsin was not used in the present study.

In the present study, the quality of the raw semen sample in terms of motility and viability was adequate for preservation of these samples by refrigeration and cryopreservation. Cryopreservation of gametes and embryos is one of the requirements for application of assisted reproductive techniques to endangered animals (Bainbridge and Jabbour, 1998; Holt and Pickard, 1999; Pukazhenthi and Wildt, 2004; Andrabi and Maxwell, 2007; Comizzoli et al., 2012).

The results from the present study indicated refrigeration for 6 h and frozen-thawed samples could be used for assisted reproductive techniques (Artificial insemination, IVF, ICSI, etc.), whereas and samples refrigerated for 24 to 30 h could only be used for ICSI procedures because of the lack of sperm progressive motility. The percentage of viable and acrosome intact spermatozoa was similar in refrigerated and frozen-thawed samples, with a lesser motility in cryopreserved samples. Cryopreservation procedures can be used without limitations of time and space, even though the semen quality was slightly less than that of refrigerated samples.

Artificial insemination is the simplest and most economical of the assisted reproduction techniques and could be used to propagate valuable animals. Although the experience in primates is limited (Wolf, 2009), there is a large amount of knowledge and experience in the management, preservation and artificial insemination with human spermatozoa. The possibility of using extender developed for human sperm refrigeration and cryopreservation facilities the process and may enable artificial insemination in primates, including the Black Crested Mangabey.

5. Conclusions

Black Crested Mangabey spermatozoa could be refrigerated for as long as 6 h or frozen-thawed with adequate viability, acrosome status and motility. The results indicate there are possibilities for further studies with the use of assisted reproductive techniques in the conservation of Black Crested Mangabey. Considering the small sample size (semen from only one animal) in the present study, further research is required to determine the scale of inter-individual variation in refrigerated and frozen-thawed sperm variables. In addition, future research should be conducted to examine the viability of preserved Black Crested Mangabey semen for *in vitro* fertilization, as well as subsequent embryo development.

Contributions

Experimental design (JG, PS, JSM). Animal management and sedation (PS). Ultrasonography and sperm recovery (ATD, JSM). Sperm assessment (JG, ATD, SNS, CM, JSM). Transmission electronic microscope evaluation (MJM). Sperm preservation (JG, SNS, CM). Data analysis and document writing (JG, JSM). All authors have approved the definitive version of this article.

Funding

This research was supported by Fundación Séneca de la Región de Murcia [grant number 20040/GERM/16].

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgements

The authors are grateful to Prof. Bill Holt (University of Sheffield) for his critical review of the manuscript and Prof. Larry Reynolds (North Dakota State University) for his help with English language. Assistance provided by all the staff from Rio Safari Elche was greatly appreciated. Dr. Tjerk ter Meulen and Emele F, Prins (GaiaZOO, the Netherlands) for facilitating information and new serum samples from mangabeys. Dr. Juan Carlos Martínez-Soto for his help with endocrine evaluation.

References

Abelló, M.T., ter Meulen, T., Prins, E.F., 2018. EAZA Mangabey Best Practice Guidelines. Parc Zoològic de Barcelona and GaiaZOO.
Andrabi, S.M., Maxwell, W.M., 2007. A review on reproductive biotechnologies for conservation of endangered mammalian species. Anim. Reprod. Sci. 99, 223–243.
Arlet, M.E., Grote, M.N., Molleman, F., Isbell, L.A., Carey, J.R., 2009. Reproductive tactics influence cortisol levels in individual male gray-cheeked mangabeys (Lophocebus albigena). Horm. Behav. 55, 210–216.
Arlet, M.E., Kaasik, A., Molleman, F., Isbell, L., Carey, J.R., Mänd, R., 2011. Social factors increase fecal testosterone levels in wild male gray-cheeked mangabeys (Lophocebus albigena). Horm. Behav. 59, 605–611.
Bader, H., 1983. Electroejaculation in chimpanzees and gorillas and artificial insemination in chimpanzees. Zoo Biol. 2, 307–314.
Bainbridge, D.R., Jabbour, H.N., 1998. Potential of assisted breeding techniques for the conservation of endangered mammalian species in captivity: a review. Vet. Rec. 143, 159–168.
Beattie, C.W., Bullock, B.C., 1978. Diurnal variation of serum androgen and estradiol-17beta in the adult male green monkey (Cercopithecus sp). Biol. Reprod. 19, 36–39.
Bedford, J.M., 1967. Observations on the fine structure of spermatozoa of the bush baby (Galago senegalensis), the African green monkey (Cercopithecus aethiops) and man. Am. J. Anat. 121, 443–459.
Bornman, M.S., van Vuuren, M., Meltzer, D.G., van der Merwe, C.A., v Rensburg, S.J., 1988. Quality of semen obtained by electroejaculation from chacma baboons (Papio ursinus). J. Med. Primatol. 17, 57–61.
Calle, P.P., Chaudhuri, M., Bowen, R., 1990. Menstrual cycle characterization and artificial insemination in the black mangabey (Cercocebus aterrimus). Zoo Biol. 9, 11–24.
Chenoweth, P.J., 2005. Genetic sperm defects. Theriogenology 64, 457–468.
Comizzoli, P., Songsasen, N., Hagedorn, M., Wildt, D.E., 2012. Comparative cryobiological traits and requirements for gametes and gonadal tissues collected from wildlife species. Theriogenology 78, 1666–1681.
Correa-Perez, J.R., Fernandez-Pelegrina, R., Zarmakoupis-Zavos, P.N., Zavos, P.M., 2003. The effect of colloid osmotic pressure in human spermatozoa exposed to hypoosmotic conditions. Andrologia 35, 117–120.
Czoty, P.W., Gould, R.W., Nader, M.A., 2009. Relationship between social rank and cortisol and testosterone concentrations in male cynomolgus monkeys (Macaca fascicularis). J. Neuroendocrinol. 21, 68–76.
Esteso, M.C., Rodríguez, E., Toledano-Díaz, A., Castaño, C., Pradiee, J., López-Sebastián, A., Santiago-Moreno, J., 2015. Descriptive analysis of sperm head morphometry in Iberian ibex (Capra pyrenaica): optimum sampling procedure and staining methods using Sperm-Class Analyzer®. Anim. Reprod. Sci. 155, 42–49.
Fleagle, J.G., McGraw, W.S., 1999. Skeletal and dental morphology supports diphyletic origin of baboons and mandrills. Proc Natl Acad Sci U S A 96, 1157–1161.
Flechon, J.E., Kraemer, D.C., Hafez, E.S., 1976. Scanning electron microscopy of baboon spermatozoa. Folia Primatol. (Basel). 26, 24–35.
Gadea, J., Molla, M., Selles, E., Marco, M.A., Garcia-Vazquez, F.A., Gardon, J.C., 2011. Reduced glutathione content in human sperm is decreased after cryopreservation: effect of the addition of reduced glutathione to the freezing and thawing extenders. Cryobiology 62, 40–46.
Gadea, J., Gumbao, D., Gomez-Gimenez, B., Gardon, J.C., 2013. Supplementation of the thawing medium with reduced glutathione improves function of frozen-thawed goat spermatozoa. Reprod. Biol. 13, 24–33.
Goodall, S.V., Chinnadurai, S.K., Kwan, T., Aitken-Palmer, C., 2018. Surgical treatment of recurrent rectal prolapse in an adult female black-crested mangabey (Lophocebus aterrimus) by colopexy. Comp. Med. 68, 80–83.
Gould, K.G., Martin, D.E., 1978. Comparative morphology of primate spermatozoa using scanning electron microscopy. II. Families cercopithecidae, Lorisidae. Lemuridae. J. Hum. Evol. 7, 637–642.
Greer, W.E., Roussel, J.D., Austin, C.R., 1968. Prevention of coagulation in monkey semen by surgery. J. Reprod. Fertil. 15, 153–155.

Griggers, S., Paccamonti, D.L., Thompson, R.A., Eilts, B.E., 2001. The effects of ph, osmolarity and urine contamination on equine spermatozoal motility. Theriogenology 56, 613–622.
Groves, C.P., 2007. The endemic Uganda Mangabey,Lophocebus ugandae, and other members of the albigena-group (Lophocebus). Primate Conserv. 22, 123–128.
Hart, J., Groves, C.P., Ehardt, C., 2008. Lophocebus Aterrimus. The IUCN Red List of Threatened Species p. e.T12310A3333720.
Hernandez-Lopez, L., Parra, G.C., Cerda-Molina, A.L., Perez-Bolanos, S.C., Diaz Sanchez, V., Mondragon-Ceballos, R., 2002. Sperm quality differences between the rainy and dry seasons in captive black-handed spider monkeys (Ateles geoffroyi). Am. J. Primatol. 57, 35–41.
Holt, W.V., Pickard, A.R., 1999. Role of reproductive technologies and genetic resource banks in animal conservation. Rev. Reprod. 4, 143–150.
Holt, W.V., O'Brien, J., Abaigar, T., 2007. Applications and interpretation of computer-assisted sperm analyses and sperm sorting methods in assisted breeding and comparative research. Reprod. Fertil. Dev. 19, 709–718.
International-Studbook, 2018. International Studbooks for Rare Species of Wild Animals in Captivity 52. International Zoo Yearbook, pp. 459–460.
Jeyendran, R.S., Van der Ven, H.H., Zaneveld, L.J., 1992. The hypoosmotic swelling test: an update. Arch. Androl. 29, 105–116.
Kyaligonza, J.K., 1998. Semen Analysis and Induction of Acrosome Reaction in Spermatozoa of Tana mangabey (*Cercocebus Galeritus*) and De Brazza's (*Cercopilhecus Neglectus*) Monkeys. Animal Physiology, University of Nairobi, Kenia, Niarobi, Kenia.
Lange, A., Bormann, C.L., 2017. Utilization of a test yolk buffer solution to maintain the integrity of semen samples for an extended period of time. Fertil. Steril. 107, e27.
Leibo, S.P., Songsasen, N., 2002. Cryopreservation of gametes and embryos of non-domestic species. Theriogenology 57, 303–326.
Levecke, B., Dorny, P., Geurden, T., Vercammen, F., Vercruysse, J., 2007. Gastrointestinal protozoa in non-human primates of four zoological gardens in Belgium. Vet. Parasitol. 148, 236–246.
Makler, A., David, R., Blumenfeld, Z., Better, O.S., 1981. Factors affecting sperm motility. Vii. Sperm viability as affected by change of ph and osmolarity of semen and urine specimens. Fertil. Steril. 36, 507–511.
Mann, D.R., Castracane, V.D., McLaughlin, F., Gould, K.G., Collins, D.C., 1983. Developmental patterns of serum luteinizing hormone, gonadal and adrenal steroids in the sooty mangabey (Cercocebus atys). Biol. Reprod. 28, 279–284.
Martin, D.E., Gould, K.G., Warner, H., 1975. Comparative morphology of primate spermatozoa using scanning electron microscopy. I. Families Hominidae, Pongidae, Cercopithecidae and Cebidae. J. Hum. Evol. 4, 287–292.
Martinez-Soto, J.C., Garcia-Vazquez, F.A., Gumbao, D., Landeras, J., Gadea, J., 2011. Assessment of two thawing processes of cryopreserved human sperm in pellets. Cryobiology 63, 131–136.
Maya-Soriano, M.J., Abelló, M.T., Taberner, E., Fernández-Bellon, H., Lopez-Bejar, M., 2012. Successful electroejaculation and cryopreservation of epididymal sperm cells from a red-capped mangabey (cercocebus torquatus torquatus). Proceedings of the International Conference on Diseases of Zoo and Wild Animals 2012 : May 16-19, 2012 157–158.
Moretti, E., Sutera, G., Collodel, G., 2016. The importance of transmission electron microscopy analysis of spermatozoa: diagnostic applications and basic research. Syst. Biol. Reprod. Med. 62, 171–183.
Morrell, J.M., Hodges, J.K., 1998. Cryopreservation of non-human primate sperm: priorities for future research. Anim. Reprod. Sci. 53, 43–63.
Mubiru, J.N., Hubbard, G.B., Dick Jr., E.J., Furman, J., Troyer, D.A., Rogers, J., 2008. Nonhuman primates as models for studies of prostate specific antigen and prostatic diseases. Prostate 68, 1546–1554.
Nallella, K.P., Sharma, R.K., Allamaneni, S.S., Aziz, N., Agarwal, A., 2004. Cryopreservation of human spermatozoa: comparison of two cryopreservation methods and three cryoprotectants. Fertil. Steril. 82, 913–918.
Nowak, R.M., Walker, E.P., Mittermeier, R.A., Rylands, A.B., Konstant, W.R., 1999. Walker's Primates of the World. Johns Hopkins University Press.
Oliveira, K.G., Leao, D.L., Almeida, D.V., Santos, R.R., Domingues, S.F., 2015. Seminal characteristics and cryopreservation of sperm from the squirrel monkey, Saimiri collinsi. Theriogenology 84, 743–749 e741.
Pradiee, J., O'Brien, E., Esteso, M.C., Castano, C., Toledano-Diaz, A., Lopez-Sebastian, A., Marcos-Beltran, J.L., Vega, R.S., Guillamon, F.G., Martinez-Nevado, E., Guerra, R., Santiago-Moreno, J., 2016. Effect of shortening the prefreezing equilibration time with glycerol on the quality of chamois (Rupicapra pyrenaica), ibex (Capra pyrenaica), mouflon (Ovis musimon) and aoudad (Ammotragus lervia) ejaculates. Anim. Reprod. Sci. 171, 121–128.
Prieto, M., Sanchez-Calabuig, M., Hildebrandt, T., Santiago-Moreno, J., Saragusty, J., 2014. Sperm cryopreservation in wild animals. Eur. J. Wildl. Res. 60, 851–864.
Pukazhenthi, B.S., Wildt, D.E., 2004. Which reproductive technologies are most relevant to studying, managing and conserving wildlife? Reprod. Fertil. Dev. 16, 33–46.
Roussel, J.D., Austin, C.R., 1967. Enzymic liquefaction of primate semen. Int. J. Fertil. 12, 288–290.
Santiago-Moreno, J., Gómez-Brunet, A., González-Bulnes, A., Toledano-Díaz, A., Malpaux, B., López-Sebastián, A., 2005. Differences in reproductive pattern between wild and domestic rams are not associated with inter-specific annual variations in plasma prolactin and melatonin concentrations. Domest. Anim. Endocrinol. 28, 416–429.
Santiago-Moreno, J., Castano, C., Toledano-Diaz, A., Esteso, M.C., Lopez-Sebastian, A., Guerra, R., Ruiz, M.J., Mendoza, N., Luna, C., Cebrian-Perez, J.A., Hildebrandt, T.B., 2013. Cryopreservation of aoudad (Ammotragus lervia sahariensis) sperm obtained by transrectal ultrasound-guided massage of the accessory sex glands and electroejaculation. Theriogenology 79, 383–391.
Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675.
Stanyon, R., Ardito, G., Lamberti, L., Bigatti, P., 1983. The banded karyotypes of Macaca fuscata compared with Cercocebus aterrimus. Folia Primatol. (Basel). 41, 137–146.
Takemura, T., Ekwalanga, M., Bikandou, B., Ido, E., Yamaguchi-Kabata, Y., Ohkura, S., Harada, H., Takehisa, J., Ichimura, H., Parra, H.J., Nende, M., Mubwo, E., Sepole, M., Hayami, M., Miura, T., 2005. A novel simian immunodeficiency virus from black mangabey (Lophocebus aterrimus) in the Democratic Republic of Congo. J. Gen. Virol. 86, 1967–1971.
Ungerfeld, R., Lopez-Sebastian, A., Esteso, M., Pradiee, J., Toledano-Diaz, A., Castano, C., Labrador, B., Santiago-Moreno, J., 2015. Physiological responses and characteristics of sperm collected after electroejaculation or transrectal ultrasound-guided massage of the accessory sex glands in anesthetized mouflons (Ovis musimon) and Iberian ibexes (Capra pyrenaica). Theriogenology 84, 1067–1074.
Unnikrishnan, R., Almassi, N., Fareed, K., 2017. Benign prostatic hyperplasia: evaluation and medical management in primary care. Cleve. Clin. J. Med. 84, 53–64.
Warner, H., Martin, D.E., Keeling, M.E., 1974. Electroejaculation of the great apes. Ann. Biomed. Eng. 2, 419–432.
Watson, P., Holt, W.V., 2001. Cryobanking the Genetic Resource: Wildlife Conservation for the Future? Taylor and Francis, London.
Wolf, D.P., 2009. Artificial insemination and the assisted reproductive technologies in non-human primates. Theriogenology 71, 123–129.
Zamboni, L., Zemjanis, R., Stefanini, M., 1971. The fine structure of monkey and human spermatozoa. Anat. Rec. 169, 129–153.