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Comparison of semen characteristics and sperm cryopreservation in common marmoset (*Callithrix jacchus*) and black-tufted-ear marmoset (*Callithrix penicillata*)

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Abstract

Background: The common marmoset (*Callithrix jacchus*) is one of the most studied Neotropical primates regarding reproduction. However, little has been reported on the black-tufted-ear marmoset (*Callithrix penicillata*), which may produce fertile hybrids with other *Callithrix*. This is the first study to evaluate sperm freezing from black-tufted-ear marmoset.

Methods: Testicles from all animals were measured, and semen was collected by penile vibrostimulation. Samples were analyzed after collection, after chilling and addition of glycerol, and after thawing.

Results and Conclusions: Fresh semen from both species was similar in many aspects. Additionally, there is a relationship between total motility, plasma membrane integrity, and acrosome integrity observed in sperm samples from both species. We managed to evaluate fresh and thawed sperm with suitable methods for use under zoo or field conditions. However, TEST egg yolk with glycerol at 4% and 6% concentration was not effective for sperm protection in both species during cryopreservation.

KEYWORDS

glycerol, penile vibratory stimulation, primate conservation, semen evaluation, TES-TRIS

1 | INTRODUCTION

Currently, some non-human primates (NHP) are classified as threatened or endangered by the International Union for Conservation of Nature (IUCN). Their populations are declining mainly because of habitat loss or hunting. Conservation of biological diversity is a major challenge nowadays, mostly because of increasing anthropic actions influence in the environment. Worldwide, the Neotropical region stands out for being among the regions presenting greatest biodiversity, however, some areas in this region are in risk of loss of biodiversity.[1-3] Of the world's 25 most endangered primates species, five occur in Neotropics.[4] In this context, the creation of genetic resources banks, consisting in the organized collection, storage, and use of germplasm—including sperm, from individuals of threatened or non-threatened species—to be used in the future, is an important tool to contribute to the conservation of these animals.[5,6]

Attempts to cryopreserve semen of NHP, especially the most commonly found species in captivity, have occurred in recent decades. However, reliable information on the most appropriate methodologies is still limited.[7] Cryopreservation of sperm from Neotropical primates has been reported for some species, including squirrel monkeys (*Saimiri sciureus*, *S. boliviensis*, *S. collinsi*,

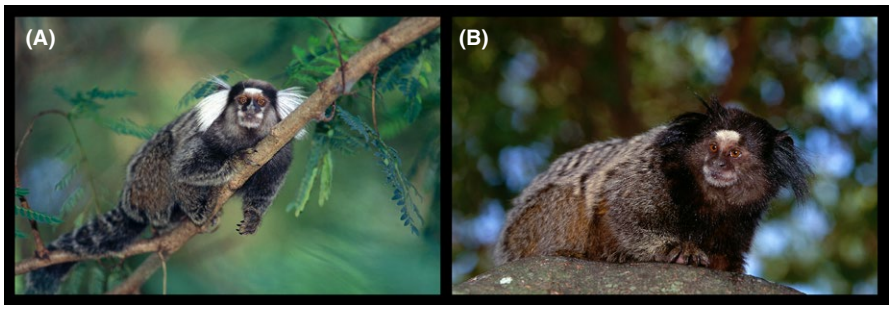

FIGURE 1 The species studied (A) common marmoset (*Callithrix jacchus*) and (B) black-tufted ear marmoset (*Callithrix penicillata*). Photos: Luis Claúdio Marigo

S. vanzolinii, *S. cassiquiarensis*, *S. macrodon)*, spider monkeys (*Ateles paniscus*, *A. marginatus*), and one species of capuchin monkey (*Sapajus apella*), tamarin (*Saguinus labiatus*), howler monkey (*Alouatta caraya*), and common marmoset (*Callithrix jacchus*).[8-18] As described for Old World monkeys,[7] the cryoprotectants used usually contain glycerol in different concentrations—ranging from 1.5% to 8%, alone or in combination with egg yolk (used most often), or soy lecithin.[8-18]

Based on sperm motility, the studies that presented the best results in frozen-thawed sperm were in *S. sciureus* and *S. labiatus*,[8,16] which presented a loss of 18.55% and 12.5% in sperm motility, respectively. The first study used a medium containing 11% of lactose, 4% of glycerol, and 20% of egg yolk, with freezing in dry ice blocks, and in the second one, epididimal samples were frozen in liquid nitrogen vapor, in a Tes-, Tris-, and egg yolk-based medium, with 5% of glycerol.

There are six species in *Callithrix* genus (*C. jacchus*, *C. penicillata*, *C. kuhlii*, *C. geoffroyi*, *C. aurita*, and *C. flaviceps*).[19] Regarding their reproductive biology, the common marmoset (*C. jacchus*) is the best known model from this genus. Despite this, there are only a few reports regarding semen cryopreservation for the common marmoset and none for the black-tufted-ear marmoset (*C. penicillata*). The objectives of this study were (a) to collect semen using the penile vibrostimulation technique and describe its characteristics from the black-tufted-ear marmoset; (b) to compare fresh semen from common marmoset and black-tufted-ear marmoset; and (c) to test two glycerol concentrations (4% and 6%) for semen cryopreservation of these two species.

2 | MATERIALS AND METHODS

2.1 | Humane care guidelines

This research complied with protocols approved by the Ethics Committee in the Use of Animals from College of Veterinary Medicine and Animal Science, University of São Paulo (no 2275/2013) and adhered to the Brazilian legal requirements (SISBIO/ICMBio/MMA no 28608-2).

2.2 | Animals and housing conditions

Common marmoset males (n = 8) and black-tufted-ear marmoset males (n = 10) (Figure 1) were maintained in captivity at the National Primate Center – CENP, located at Ananindeua city, state of Pará, Northern Brazil. Animals were housed in enclosures (1.5 m × 0.9 m × 2 m) positioned in a north-south orientation to receive 12 hours of natural light each day, with groups' compositions typically consisting of a male, a female, and their young prole. The average temperature was 33°C, and humidity was 85%. Evaluated males were adults, with a mean age of 4.5 years for both species, ranging from 3 to 9 years in common marmoset and from 3 to 7 years in black-tufted-ear marmoset, all with a proven history of fertility (presence of offspring). The diet consisted of daily fresh fruits, vegetables, and primate pellets (Callitrichidae P25 Megazoo®, Betim, MG, Brazil); animals also received beetle larvae twice a week, and water was available ad libitum.

2.3 | Testicular morphometry and semen collection

During procedures, an expert animal keeper physically restrained the animals. To evaluate testicular morphometry, animals were kept in the dorsal decubitus position, and both testes were measured with a digital caliper. We determined testicular volume by Lambert's empirical formula: volume = length × width × height × 0.71,[20] already applied for other Neotropical primate, Goeldi's monkey (*Callimico goeldii*).[21] Total testicular volume was calculated by adding the volumes of the right and left testicles. We measured the scrotal circumference with a measuring tape.

After inspection and palpation of testicles, genital area was washed with liquid soap (hypoallergenic glycerinated baby soap, Casa Granado® Laboratórios, Farmácias e Drogarias SA, Belém, PA, Brazil), rinsed with water at 37°C, and dried with soft paper, to minimize animal discomfort.

We performed all semen collections in the morning before feeding, and the interval between collections from the same male was at least 1 week. Semen was collected from February to May in 2013 (rainy season).

During semen collection, animals remained restrained by the hands of the assistant; one hand held the head, thorax, and forelimb of the animal, and the other hand contained the left leg and tail, exposing the genital region to receive the artificial stimuli from the vibrostimulation apparatus.

Semen samples from the common marmoset (n = 18) and the black-tufted-ear marmoset (n = 6) were collected by penile vibrostimulation technique (PVS) using a vibrostimulation apparatus

(FertiCare® - Multicept ApS, Rungsted, Denmark), with modifications of a published protocol[22] previously used for common marmoset.[23] The stimuli applied for obtaining the ejaculates varied according to the responses of individuals and between species. For common marmoset, frequency of 70-80 Hz and amplitude of 1.0 mm, and for black-tufted-ear marmoset, frequency of 75-85 Hz and amplitude of 1.0-1.5 mm. Semen collection procedure consisted of repeated series of two minutes of stimulation, followed by resting phases of 30 seconds, during which animals received rewards, such as beetle larvae, and bananas. If a male did not ejaculate after 20 minutes, the procedure was ended and was repeated after a period of at least 1 week.

2.4 | Semen evaluation

The same investigator conducted all evaluations in the same manner for both species. Immediately after ejaculation, semen pH was measured using a pH strip (Merck®, Darmstadt, Germany). Then, a fixed volume of 200 μL of TALP-HEPES medium at 37°C was added to the collection tube, to increase the sample volume and to allow both fresh semen analysis and posterior semen storage in liquid nitrogen. After dilution, we determined the semen volume by weighing the tube containing the diluted semen using a precision scale (Chyo JK-180®, YMC Co. Ltd., Kyoto, Japan), and then the tube's weight and the amount of extender used were subtracted from the total weight, assuming the density of semen to be 1 g/mL in marmosets, equal to that of humans.[21,24]

Diluted semen samples were maintained at 37°C during the period of analyses. We evaluated semen to determine total and forward progressive motility, sperm concentration, plasma membrane integrity, acrosome integrity, mitochondrial activity, and morphology. Those evaluations were performed in three moments: (a) immediately after collection (fresh semen); (b) after refrigeration and glycerol addition (cooled semen); and (c) after thawing the sample frozen for more than a month (thawed semen), in the presence of 4% or 6% glycerol—sperm concentration was not reevaluated for 2 and 3.

The first analysis performed was the percentage of motile sperm and forward progressive sperm motility. In order to quantify sperm motility, 10 μL of semen was placed in a pre-warmed (37°C) glass slide with a coverslip and were evaluated under a light microscope, at ×400 magnification. Sperm concentration was determined in a Neubauer chamber after dilution of 10 μL of semen in 90 μL of 10% formol saline solution.

The percentage of sperm with intact plasma membrane was determined after evaluation of a smear of semen stained with eosin-nigrosin staining (VitaScan®; Lucron Bioproducts, Gennep, Netherlands). For acrosome integrity assessment, we used the fast-green/rose-bengal staining, and smears were prepared by the addition of 5 μL of diluted semen to 5 μL of stain solution, with incubation for 90 seconds. In both cases, we prepared smears on a pre-warmed (37°C) glass slide and 200 sperm were assessed, at ×1000 magnification.[23] For the evaluation of acrosome integrity in thawed samples, we diluted semen with TALP-HEPES at a ratio of 1:1, prior to addition of staining, in order to reduce the presence of debris (a reaction of staining solution with egg yolk contained in the cryoprotectant) in background.[25] Both stainings were validated for common marmoset semen by Valle et al[23]

Mitochondrial activity was evaluated by 3,3′diaminobenzidine (DAB) staining.[26] Semen was placed in an amber tube with the same quantity of a DAB solution and incubated for 1 hour at 37°C. A smear was prepared and fixed in 10% formalin for 10 minutes. Sperm cells were observed under oil immersion using a phase contrast microscope (Nikon® Corporation, Tokyo, Japan), and 200 cells were evaluated and classified into four different classes: Class I with 100% of the midpiece stained; Class II with more than 50% of the midpiece stained; Class III with less than 50% of the midpiece stained; and Class IV with no staining of the midpiece, which indicates full, medium, low, and no mitochondrial activity, respectively.[26] (Figure 2).

Sperm morphology was performed by the evaluation of 200 spermatozoa, through wet mount, with differentiation between the cells presenting normal morphology and those presenting abnormalities. Sperm alterations were classified into major and minor defects,[27] and their sum is presented as total defects.

2.5 | Semen refrigeration and cryopreservation

After performing the analyses with fresh semen, we diluted the remaining samples with TES-TRIS clarified egg yolk extender without glycerol (Fraction A) and placed into two Eppendorf tubes. The volume of extender used was calculated based on the sperm concentration value of the sample, to achieve a final concentration of 25×10^6 spermatozoa/mL per straw. When the number of sperm was not sufficient to achieve this concentration, only one straw per treatment was frozen. The tubes with the diluted sample were placed into a refrigerator at 4°C inside an acrylic box containing water at 25°C (150 mL), for 2 hours, until samples reached 4°C. After this period, samples were diluted further with slow addition (dropwise) of the medium TES-TRIS clarified egg yolk, with glycerol 8% and 12% (Fraction B), to achieve final concentrations of 4% and 6% glycerol, one tube for each concentration. At this point, analyses were performed as described above (cooled semen).

Immediately after preparation of the slides for the analyses, samples were aspirated into French straws, which had been previously cut to reach a final capacity of 100 μL. Straws were transferred to liquid nitrogen vapor, where they remained for 10 minutes. Sequentially, they were immersed and stored in liquid nitrogen, following a protocol described for the common marmoset.[15]

2.6 | Semen thawing

Samples were thawed by immersion in water at 37°C for 15 seconds, then transferred to an Eppendorf tube at 37°C and maintained at this temperature until the completion of the last evaluation. We performed the analyses at 10, 40, and 80 minutes after thawing (thawed semen).

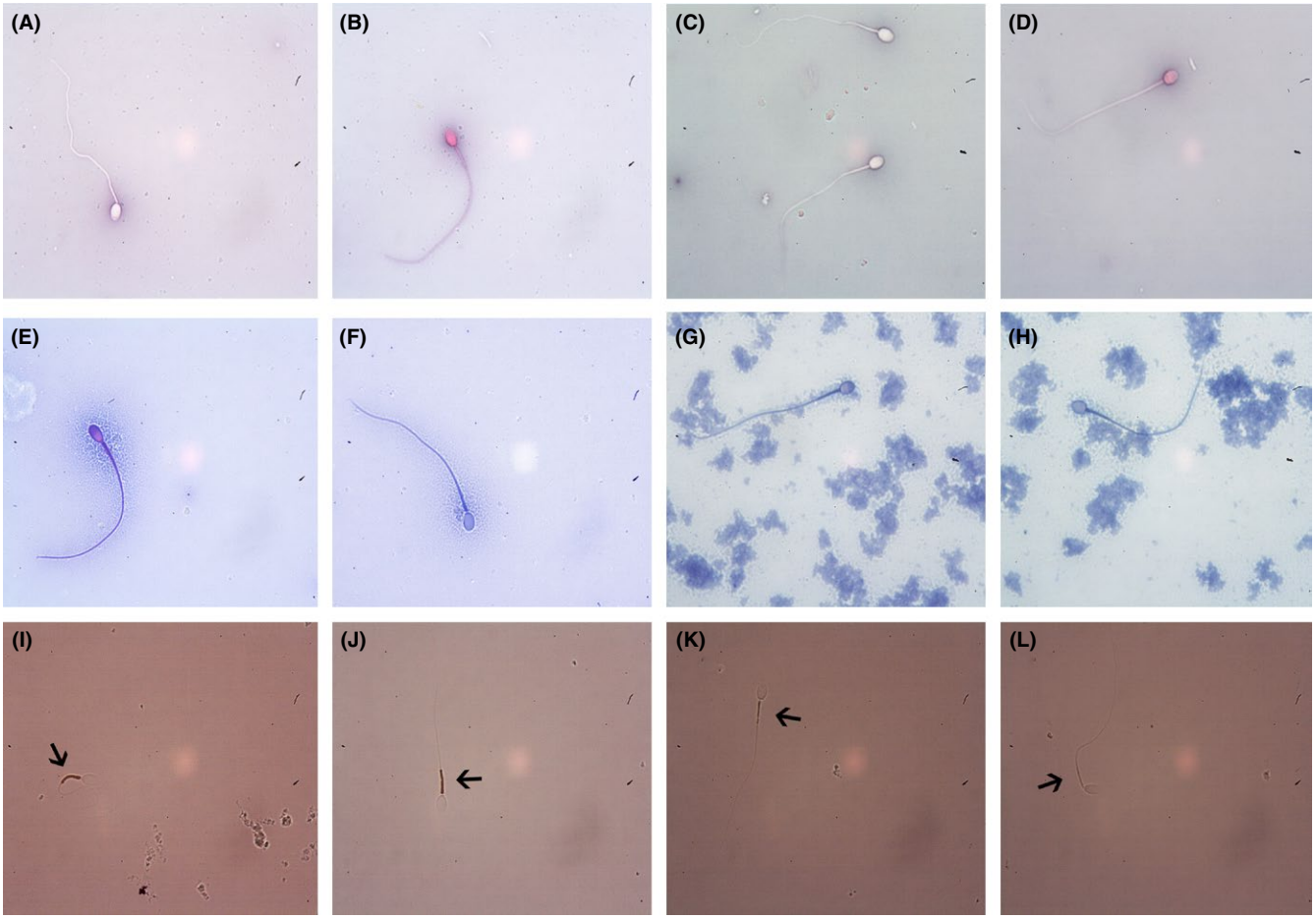

FIGURE 2 Methods used to evaluate common marmoset (*Callithrix jacchus*) and black-tufted ear marmoset (*Callithrix penicillata*) sperm. Photomicrographs of membrane integrity in spermatozoa assessed by eosin-nigrosin where (A) intact membrane was not stained, and (B) those with damaged membrane stained pink in common marmoset semen; (C) intact membrane and (D) damaged membrane in black-tufted ear marmoset semen. Acrosome integrity was assessed by fast-green/rose-bengal, where (E) in intact acrosome, the acrosomal region stained purple, which was darker than the post-acrosomal region, that stained blue, and (F) sperm with damaged acrosome stained the complete head in blue, in common marmoset fresh semen; (G) intact acrosome and (H) damaged acrosome in black-tufted ear marmoset thawed semen, with visible debris from reaction between staining solution and cryoprotectant egg yolk. Mitochondrial activity of common marmoset (*C. jacchus*) sperm, assessed by 3,3′diaminobenzidine, (spermatozoa midpiece evidenced by black arrows), (I) class I denote spermatozoa with 100% active mitochondria, (J) class II, more than 50% active mitochondria, (K) class III, less than 50% active mitochondria and (L) class IV, 0% active mitochondria. Photos: Paloma Rocha Arakaki

2.7 | Statistical analysis

Data were evaluated using SAS System for Windows (SAS Institute Inc., Cary, NC, USA). In order to compare the effect of two different glycerol concentrations (4% and 6%) on spermatic quality, data were tested for normality of residues (normal distribution) and homogeneity of variances using the Guided Data Analysis application. Thus, least significant difference (LSD) and student's *t* tests were used for parametric data as well as the Wilcoxon test was used for non-parametric data. For the results description, untransformed means and their respective standard errors were shown. A probability value of $P < 0.05$ was considered statistically significant. The response variables were also submitted to the correlation tests of Pearson and Spearman to the parametric and non-parametric variables, respectively.

3 | RESULTS

3.1 | Success rate of PVS technique

A total of 56 semen collection attempts were performed in both species. In the common marmoset males (n = 8), 18 ejaculates were obtained from 26 attempts, and the success rate was 69.23%. The success rate of PVS for the black-tufted-ear marmoset males (n = 10) was 20%, with six ejaculates from 30 attempts.

3.2 | Testicular morphometry

All males from both species presented testes with normal consistency and mobility. Common marmoset mean testicular volume was 1880.22 mm^3, ranging from 1498.1 mm^3 to 2444.66 mm^3.

TABLE 1 Mean (±standard error), with comparisons of the testicular morphometry mean values from common marmoset and black-tufted-ear marmoset

	Common marmoset (n = 8)	Black-tufted-ear marmoset (n = 10)	*P*
Right testicle			
Length (mm)	14.26 ± 1.05	16.35 ± 1.21	0.2332
Width (mm)	8.81 ± 0.58	11.03 ± 1.30	0.1150
Height (mm)	7.85 ± 0.29	10.47 ± 1.06	0.0210*
Volume (mm^3)	705.07 ± 75.04	1420.05 ± 338.55	0.0358*
Left testicle			
Length (mm)	15.98 ± 0.88	15.06 ± 1.57	0.5943
Width (mm)	9.71 ± 0.79	10.51 ± 0.99	0.5454
Height (mm)	10.49 ± 1.05	9.03 ± 0.85	0.3529
Volume (mm^3)	1108.89 ± 222.92	1098.27 ± 311.26	0.9780
Total volume (mm^3)	1880.22 ± 176.25	2518.31 ± 645.56	-
Scrotal circumference (cm)	5.28 ± 0.14	5.70 ± 0.18	0.0977

* indicates significant differences between species ($P < 0.05$).

TABLE 2 Mean (±standard error) values of seminal pH, sample volume (μL), sperm concentration ($\times 10^6$ sperm/mL), total sperm motility (%), forward progressive motility (%), plasma membrane integrity (intact membrane, %), acrosome integrity (intact acrosome, %), and mitochondrial activity (DABI, II, III and IV, %) of fresh semen from common marmoset and black-tufted-ear marmoset

	Common marmoset's ejaculates (n = 18)	Black-tufted-ear marmoset's ejaculates (n = 6)	*P*
pH	7.48 ± 0.05	7.53 ± 0.08	0.5753
Volume	15.61 ± 3.51	16.83 ± 4.14	0.8545
Sperm concentration	1238.58 ± 191.21	1473.85 ± 216.23	0.5163
Total motility	51.11 ± 4.19	56.67 ± 2.11	0.2491
Progressive motility	35.00 ± 4.80	41.67 ± 1.67	0.2041
Intact membrane	48.56 ± 2.84	62.83 ± 6.90	0.0330*
Intact acrosome	66.56 ± 2.81	76.67 ± 5.10	0.0891
DABI	44.67 ± 4.16	29.17 ± 5.78	0.0649
DABII	29.22 ± 3.32	43.83 ± 5. 48	0.0369*
DABIII	4.00 ± 0.73	3.00 ± 1.60	0.5256
DABIV	22.11 ± 4.76	24.00 ± 8.35	0.8452

* indicates significant differences between species ($P < 0.05$).

For black-tufted-ear marmoset, the mean testicular volume found was 2518.31 mm^3, ranging from 1079.00 mm^3 to 4210.66 mm^3. Significant difference was found for right testicle height and volume between both species (Table 1).

3.3 | Evaluation and comparison of fresh semen from common marmoset and black-tufted-ear marmoset

We obtained 18 ejaculates of common marmoset from six subjects (three ejaculates per animal) and six ejaculates of black-tufted-ear marmoset from four subjects (three ejaculates from the same male and the other ejaculates from three different males). Results from the comparison of seminal characteristics between both species are shown in Table 2.

Values of sperm morphology are shown in Table 3, which presents the percentages of spermatozoa with abnormal morphology, and the percentages of each major and minor defects found in fresh semen from both species. The most common morphologic change observed was strongly coiled tail or folded tail. There was no significant difference between species in the percentage of total abnormal sperm.

The correlation analysis revealed positive correlations between total motility and plasma membrane integrity for both common marmoset and black-tufted-ear marmoset ($r = 0.5316$, $P = 0.0232$ and $r = 0.8402$, $P = 0.0363$), and positive correlations between total motility and acrosome integrity ($r = 0.5948$, $P = 0.0092$ and $r = 0.8281$, $P = 0.0418$). Negative correlations were found between total motility and the percentage of spermatozoa with abnormal morphology in both species ($r = -0.5925$, $P = 0.0096$ and $r = -0.8281$, $P = 0.0418$, for common marmoset and black-tufted-ear marmoset, respectively).

3.4 | Evaluation of the cryopreservation effect on common marmoset semen characteristics

The analysis of glycerol concentration effect (4% or 6%) on semen was performed in four moments: (a) shortly after glycerol addition, when semen had already been cooled; (b) 10 minutes after thawing;

TABLE 3 Mean (±standard error) of percentages of sperm with major and minor pathologic defects in fresh semen from common marmoset and black-tufted-ear marmoset, and their probability values (*P*)

Morphological sperm classification (%)	Common marmoset's ejaculates (n = 18)	Black-tufted-ear marmoset's ejaculates (n = 6)	*P*
Free pathological head	0.22 ± 0.10	0	0.0416*
Pear-shaped head	0.05 ± 0.05	0	0.3313
Pouch formation	0.05 ± 0.05	0	0.3313
Coiled tail around head	1.38 ± 0.44	0.50 ± 0.34	0.2801
Strongly coiled or folded tail	19.61 ± 1.65	17.67 ± 1.84	0.5344
Bent tail with droplet	3.50 ± 1.00	5.33 ± 1.45	0.3532
Midpiece defects	0.50 ± 0.14	0.16 ± 0.16	0.2336
Proximal droplet	6.89 ± 1.32	5.83 ± 1.40	0.6698
Double forms	0.11 ± 0.08	0.33 ± 0.21	0.2232
Total major defects	32.33 ± 1.60	29.83 ± 2.61	0.4351
Narrow head	0.16 ± 0.09	0	0.0827
Giant head	0.38 ± 0.18	0.33 ± 0.21	0.8726
Short head	0.05 ± 0.05	0.16 ± 0.16	0.4159
Free normal head	11.11 ± 2.60	12.16 ± 2.71	0.8287
Simple bent tail	19.22 ± 1.69	26.00 ± 0.93	0.0020*
Coiled tail	1.83 ± 0.54	0.33 ± 0.21	0.0178*
Retro implantation	0.05 ± 0.05	0	0.3313
Oblique implantation	0.05 ± 0.05	0	0.3313
Distal droplet	0.05 ± 0.05	0	0.3313
Free normal head + midpiece	0.50 ± 0.14	1.00 ± 0.68	0.5035
Total minor defects	35.72 ± 2.38	40.00 ± 2.61	0.3433
Total abnormal sperm	68.05 ± 2.50	69.83 ± 2.84	0.7071

* indicates significant differences between species ($P < 0.05$).

(c) 40 minutes after thawing and (d) 80 minutes after thawing. Results are shown in Tables S1 to S4. In cooled semen, 4% glycerol obtained better performance regarding mitochondrial activity Class II (DABII) and Class IV (DABIV) ($P = 0.0085$ and $P = 0.0262$, respectively) when compared to 6% glycerol. No significant difference between glycerol concentrations was found in the analyses performed at 10 minutes after thawing. At 40 minutes after thawing, the values of total motility ($P = 0.0225$), plasma membrane integrity ($P = 0.2025$), and total abnormal morphology sperm cells ($P = 0.0230$), presented better results in 4% glycerol. About 4% glycerol also presented better performance in the acrosome integrity ($P = 0.0200$) in the analysis at 80 minutes after thawing.

We also carried out comparisons on seminal characteristics between the five moments of analyses (fresh semen, cooled semen, and thawed semen at 10, 40, and 80 minutes) for both glycerol concentrations (4% and 6%; Figure 3). Sperm total motility significantly decreased from fresh semen to cooled semen (41.85% in 4% glycerol and 46.74% in 6% glycerol). Except for the percentage of morphologically abnormal sperm cells, all the evaluated parameters—total sperm motility, forward progressive motility, plasma membrane integrity, acrosome integrity, and mitochondrial activity were negatively affected by freezing, regardless of glycerol concentration (4% or 6%).

3.5 | Evaluation of the cryopreservation effect on black-tufted-ear marmoset semen characteristics

The analysis of glycerol concentration effect (4% or 6%) on semen was performed as described for common marmoset. Results are shown in Tables S5-S8. No significant difference between glycerol concentrations was found in cooled semen. The 4% glycerol concentration presented better results regarding progressive motility ($P = 0.0464$) 10 minutes after thawing and at 80 minutes after thawing regarding total motility ($P = 0.0422$) and progressive motility ($P = 0.0250$).

Like performed for common marmoset, comparisons on seminal characteristics between the moments of analyses for both glycerol concentrations (4% and 6%) were done (Figure 4). As observed for common marmoset, sperm motility significantly decreased from fresh semen to cooled semen in both glycerol concentrations (57.36% decrease in total motility for 4% glycerol and 63.24% for 6% glycerol), and in both glycerol concentrations, semen quality was negatively affected by freezing.

4 | DISCUSSION

Reproductive studies with Neotropical species present a challenge for primatological issues, especially due to difficulty and cost of

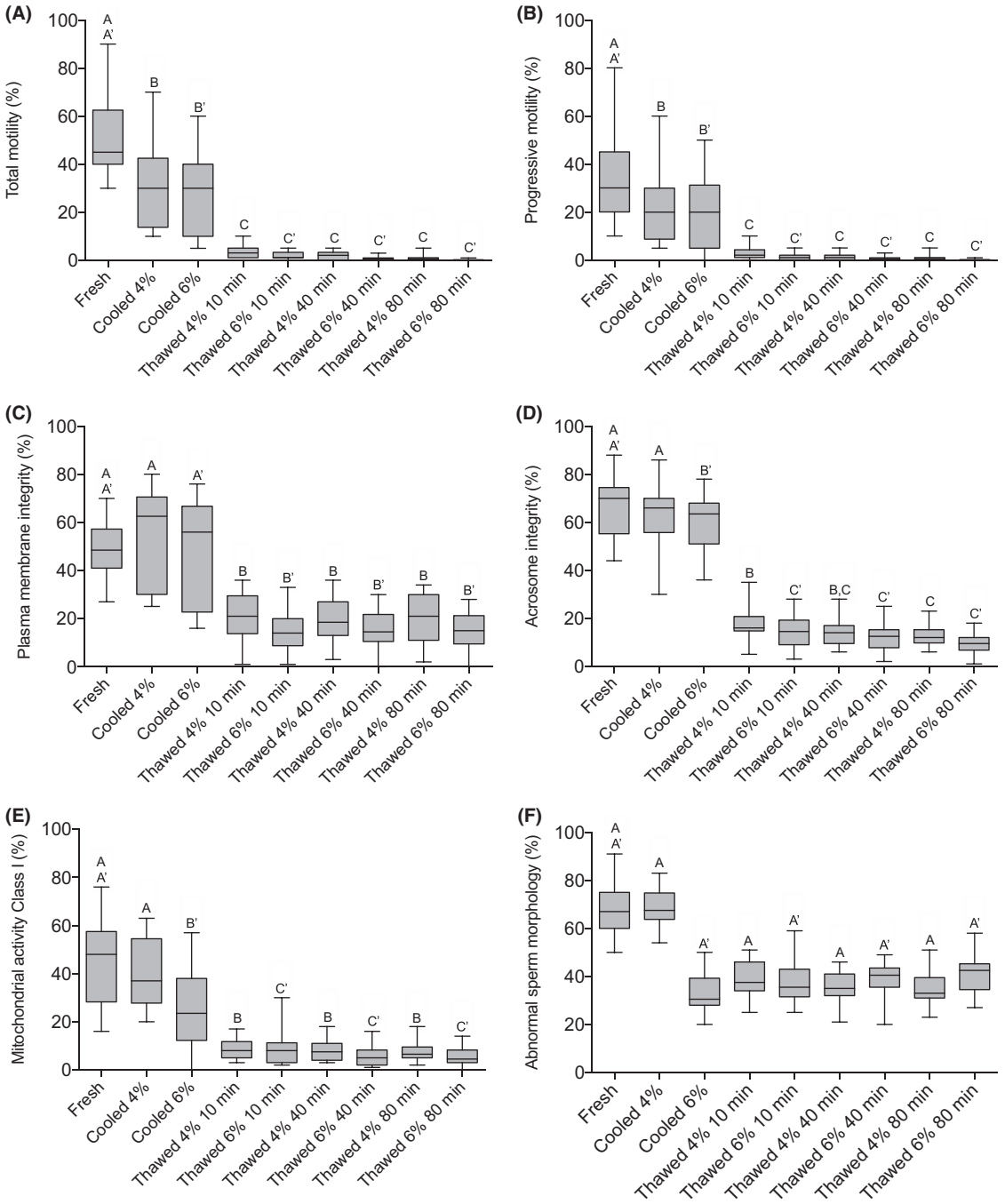

FIGURE 3 Sperm parameters of fresh, cooled, and thawed semen at 10, 40, and 80 min after thawing using 4% or 6% glycerol, in common marmoset samples. A, Total sperm motility, (B) progressive motility, (C) plasma membrane integrity, (D) acrosome integrity, (E) mitochondrial activity Class I, and (F) abnormal sperm morphology. Different uppercase letters indicate significant differences among groups within each parameter ($P < 0.05$). $^{A\text{-}C}$ 4% glycerol, $^{A'\text{-}C'}$ 6% glycerol

keeping specimens in captivity. In this context, this was the first study describing the use of PVS for semen collection in black-tufted-ear marmoset, as well as semen freezing of this species, besides the comparison of fresh semen from common marmoset and black-tufted-ear marmoset. The results discussed here were based on Neotropical primates' data.

Semen collection by PVS technique offers the advantages of not requiring sedation, causes little discomfort[28] and produces ejaculates of increased quality.[28,29] Success rate using PVS are reported in common marmoset, with values of 89.2% and 83.3%,[28,30] higher than those found in our study (69.23%). Subjects of both species were positively reinforced during semen collection period, while maintaining consistent conditions during procedures. There was no evidence of discomfort during handling, which, if present, would justify the lower success rate, especially for black-tufted-ear marmoset. It is known that significant differences among species with respect

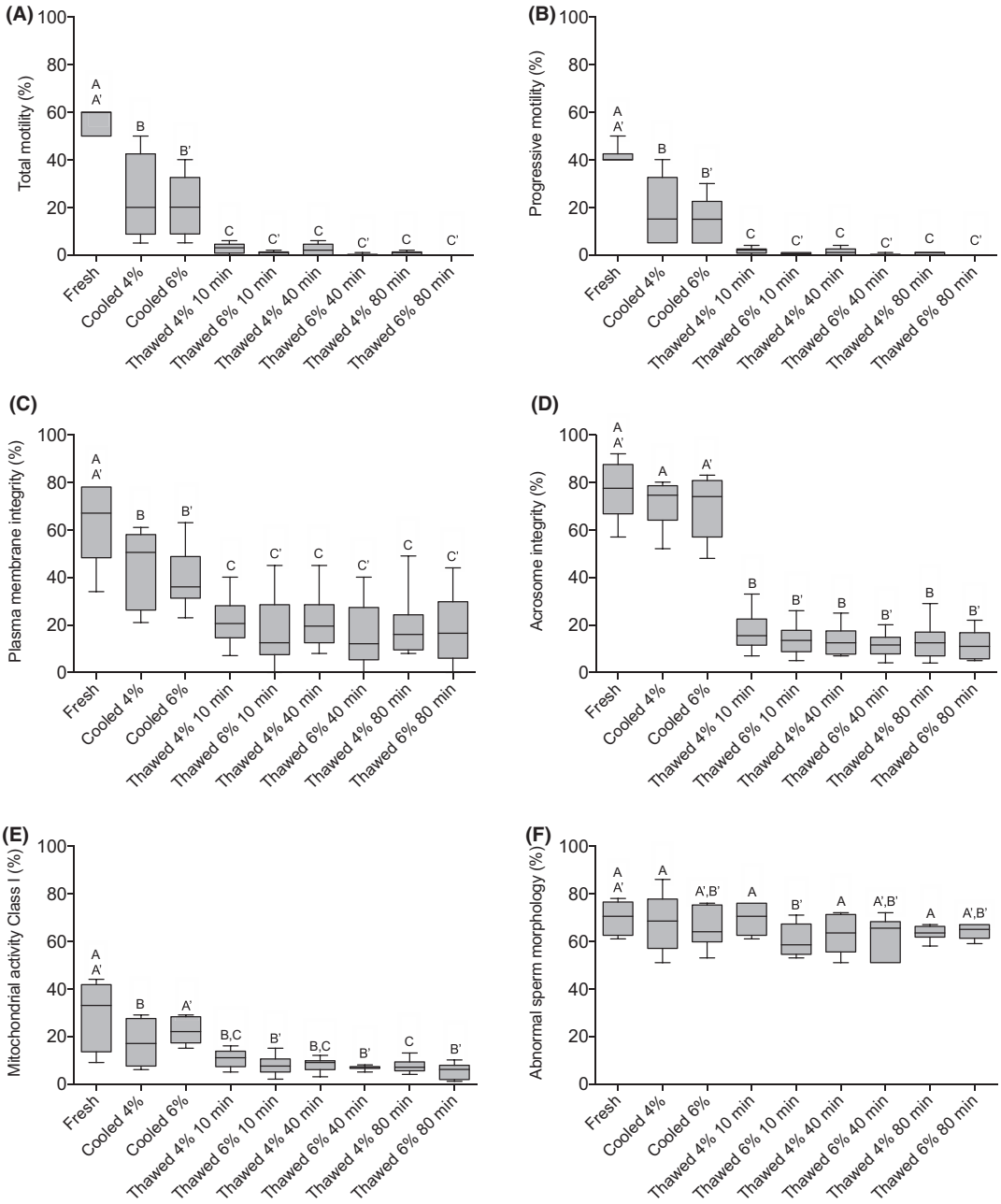

FIGURE 4 Sperm parameters of fresh, cooled, and thawed semen at 10, 40, and 80 min after thawing using 4% or 6% glycerol, in black-tufted-ear marmoset samples. A, Total sperm motility, (B) progressive motility, (C) plasma membrane integrity, (D) acrosome integrity, (E) mitochondrial activity Class I, and (F) abnormal sperm morphology. Different uppercase letters indicate significant differences among groups within each parameter ($P < 0.05$). $^{A\text{-}C}$ 4% glycerol, $^{A'\text{-}C'}$ 6% glycerol

to reproduction aspects occur, even within the same genus.[31] One of these aspects may be the response to PVS stimuli, when different species of the same genus present different reactions, as also observed for lion tamarins, *Leontopithecus* [Paloma Rocha Arakaki, PRA unpublished data]. In addition to the differences between species, differences in animal conditioning, housing, management, and other variables, we do not control that affect the different populations could also influence the success of PVS.

One of the objectives of the work was the application of PVS technique for semen collection. The success rate for PVS was lower than previously reported for common marmoset[28,30], especially for black-tufted-ear marmoset, which led to results based in a small amount of ejaculates. Studying wild animals, even those in captivity, is challenging. The access to the subjects is limited compared to domestic animals, due to individuals' availability, daily management of the institutions housing these animals, in addition to the limitation of handling them, in order to minimize the stress caused by the procedures.

Testicular volume found for common marmoset differs significantly from that found for black-tufted-ear marmoset regarding the

right testicle. In a study with free-living common marmoset with a different methodology for calculating testicular volume, the mean right testicle volume described for breeding males was 750 mm^3,[32] similar to the values found for the same species in the present study. However, common marmoset left testicles and both testes of black-tufted-ear marmoset presented higher values. Since testicular volume in adult common marmoset depends on the reproductive status of the male and its social position in the group,[32] these differences should not be expected; regardless of captivity or free-living animals, all animals studied were breeding males. There is no report on black-tufted-ear marmoset testicular volume. The small number of animals evaluated may have influenced the results; therefore, further measurements of testicular parameters from more males besides yearlong measurements should be taken to fully explain these variations.

In all seminal parameters evaluated, only the mean value of sperm with intact plasma membrane was significantly different between species. Regarding seminal volume, results were lower than that reported for common marmoset—mean of 26.59 μL,[30] and higher than the mean described in black-tufted-ear marmoset, 7.02 μL.[33] In all cases, semen was collected by the same technique, while volume measurements were performed using different methods. In former studies, semen volume was obtained with positive aspiration or automatic pipettes. We measured seminal volume gravimetrically. Assuming that semen density is 1 g/mL, seminal volume measurement by weighing is a viable alternative, especially with viscous samples, where accurate pipetting is more difficult.[34]

In the present study, seminal volume obtained had an average of 15 μL, in both species. Even with dilution in TALP-HEPES, the final sample volume available was still small. Additional care when working with these features should be taken, from the careful selection of which protocols to use in semen analysis, to careful handling of the sample such as during pipetting and dilution in extenders.

Total motility presented lower mean values than those reported—82.07% and 81.2% for common marmoset and black-tufted-ear marmoset, respectively.[30,33] In a third study with common marmoset, the mean value reported was 59.6%,[22] which is closer to that described in our work. Many factors could affect total motility, such as nutritional factors, variable seminal composition mainly due to prostatic and seminal vesicles contribution and environmental conditions during semen handling.[35-37] It is also important to consider that in all cases total motility was evaluated after semen dilution—medium composition and dilution factor could affect sperm motility.

For sperm concentration, studies with common marmoset[22,30] reported values (1154.2×10^6 and 1062.59×10^6 sperm/mL) similar to those observed in the present study. Based on these results, it is possible to suggest that these values could be considered as the normal parameter for the species when semen is collected by PVS. For black-tufted-ear marmoset, the mean value found was higher than that described for the species, 29.53×10^6 sperm/mL.[33]

The mean value of sperm with intact plasma membrane was lower than those described for common marmoset (74.6% and 84.7%) and for black-tufted-ear marmoset (84.7%).[22,30,33] Regarding acrosome integrity, the mean value found for common marmoset was lower than previously reported (84.6%) and close to that reported for black-tufted-ear marmoset (78.9%).[30,33] Considering all factors affecting seminal parameters discussed so far, and the fact that these are wild species, it is reasonable to expect the differences found. However, in light of the relationship between total motility, plasma membrane integrity, and acrosome integrity observed in this study for both species, it might be expected that our results for these variables are reliable.

It is interesting to note that, when comparing results found for testicular volume in both species and, seminal volume and sperm concentration for black-tufted-ear marmoset with values reported in literature, our results present higher values for these three parameters. We studied captive animals, while the values reported were obtained from free-living[32] or recently captured wild animals.[33] One explanation is that the captive condition, by the fact of conditioning greater proximity between several males at the same time, may have influenced the greater values found by us.

The freezing strategy proposed here (cryopreservation of semen with TEST egg yolk extender, glycerol at 4% and 6%, and the present freezing protocol) was an attempt to improve the semen cryopreservation protocol, based on previous work with primates, human, and bovine sperm.[15,38-41] However, it was unable to preserve sperm quality. All variables—except sperm morphology, presented degradation of semen quality after refrigeration and addition of glycerol and/or thawing. When comparing glycerol concentrations, 4% presented better results.

Although it is known that during cryopreservation semen is subjected to steps that may potentially harm sperm, such as changes in temperature, osmotic stress due to exposure to cryoprotectants molar concentrations and formation/dissolution of ice crystals in the extracellular medium,[42,43] it is difficult to precisely determine which factors caused the decline in thawed sperm quality. One explanation could be the addition of glycerol after cooling performed in this study, based on the fact that glycerol, despite being widely used in NHP semen cryopreservation, has a toxic and harmful effect on sperm quality.[42-44] Another possibility is that, as initial sperm quality also influences the subsequent ability to withstand the stresses associated with freezing and thawing,[43] the relatively low values presented in evaluations of fresh semen may have led to even lower values in thawed semen.

This study presents novel information in black-tufted-ear marmoset seminal traits and provides knowledge to base further studies for semen cryopreservation in *Callithrix* genus. Semen characteristics of other species from this genus, including those vulnerable and threatened with extinction, should be carefully evaluated for similarities and differences with the characteristics described here. Also, an efficient semen freezing protocol should be developed for these species.

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