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Evaluation of sperm quality in successive regular collections from captive black-and-gold howler monkeys (*Alouatta caraya*)

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Abstract

The black-and-gold howler monkey serves as a suitable model for development of assisted reproductive techniques (ARTs), which may later be applied in threatened species of the same genus. The objectives of this study were to evaluate semen characteristics of black-and-gold howler monkeys (*Alouatta caraya*), with emphasis on mitochondrial activity and acrosome integrity, using staining methods that are suitable for use under field conditions. Twenty six semen samples were collected by rectal probe electroejaculation from six adult captive males from the National Primate Center (CENP), Ananindeua, PA, Brazil. The following parameters were evaluated in each semen sample: volume, pH, concentration, plasma membrane integrity, acrosome integrity, and mitochondrial activity. Both Spermac stain and the Simple Staining Method were suitable for acrosome evaluation. Diaminobenzidine needs further investigation to improve its application for this species. Overall semen quality improved from the first collection to the fifth collection. This highlights the importance of repeating semen collections when evaluating a male for use in ARTs. This research brought novel information on semen characteristics of *Alouatta caraya* which will aid further studies on the application of ARTs in this and other primate species.

Keywords: acrosome integrity, diaminobenzidine, mitochondrial activity, semen, Simple Staining Method.

Introduction

There are 19 species and subspecies of the *Alouatta* genus (Rylands and Mittermeier, 2009), popularly known as the howler monkey. According to the IUCN Red List of Threatened Species (International Union for Conservation of Nature – IUCN, 2011), four species are under some level of risk. The black-and-gold howler monkey (*Alouatta caraya*) is classified as Least Concern (Fernandez-Duque *et al*., 2008; IUCN, 2011) and is readily available in captivity, which makes it a suitable model for the development of assisted reproductive techniques (ARTs), which may later be applied in threatened species of the same genus (Moreland *et al*., 2001). There are two previous reports on semen characteristics for this species (Moreland *et al*., 2001; Valle *et al*., 2004), but neither of them evaluated mitochondrial activity or acrosome integrity. Mitochondrial activity is involved in cellular respiration and energy production in the sperm cell, especially when there is modification of the energy metabolism without modification of plasma membrane (Kato *et al*., 2002). Moreover, cytochrome c oxidase is an enzyme responsible for the production of energy in the mitochondria. Based on these concepts, an assay for the quantitative and qualitative analysis of the activity of this enzyme was developed by Hrudka (1987), through the use of the chromogen 3,3'-diaminobenzidine (DAB). This technique has been previously used in two Neotropical Primate (NP) species, *Callithrix jacchus* (Valle, 2007) and *Callithrix penicillata* (Massaroto *et al.,* 2010).The acrosome contains enzymes involved in the fertilization process, which are responsible for the penetration of spermatozoa in the oocyte (Garner and Hafez, 2004). There are a few different techniques to assess acrosome status which have been described for NP semen, including fluorescent probes (O'Brien *et al*., 2003; Valle *et al*., 2008), the commercial kit Spermac® (Stain Enterprises, Onderstepoort, South Africa; Valle, 2007) and the Simple Staining Method developed by Pope *et al*. (1991; Paz *et al*., 2006a, b; Valle *et al*., 2008; Massaroto *et al*., 2010; Arakaki *et al*., 2011). However, fluorescent probes are expensive and require expensive equipment, which makes them unsuitable for use under field conditions. The objectives of this study were to evaluate semen characteristics of black-and-gold howler monkeys and the influence of successive regular collections, with emphasis on mitochondrial activity and acrosome integrity, using staining methods that are suitable for use under field conditions.

Materials and Methods

The study was conducted at the National Primate Center (CENP), Ananindeua, Pará, Brazil, during the spring of 2010.

Animals

Six adult captive *Alouatta caraya* males from CENP were used in this study. Three of the animals

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were born in captivity - BAB (5 years old), BAA (8 years old), and ABJ (16 years old) - and three were born in the wild - BAC (estimated age: 6 years old), AFZ (estimated age: 16 years old), AAF (estimated age: 16 years old). Three of the males were housed in pairs (one male and one female), two were housed in a trio (two males and one female) and one was housed individually. Cages measured 2.40 x 2.85 x 3.60 m (height x width x length) when there were two or more animals and 70 x 60 x 70 cm, when there was only one animal. Animals were subjected to natural daylight time and climate of the Amazon region and fed primate chow (P-18, Megazoo - Betim, MG, Brazil), fruits, vegetables and amino acid supplement.

Testicular morphometry

Before the first semen collection from each male, scrotal perimeter and testicular measures were taken including length, width, and height. Testicular measures were used to determine testicular volume through Lambert's empiric formula "volume = length x width x height x 0.71" which, according to Hsieh *et al*. (2009), is the best formula to calculate testicular volume in humans. Total volume was calculated by adding the volumes of the right and left testicles.

Semen collection and processing

Males were separated from females 24 h prior to semen collection to avoid copulation during this period. Animals were anesthetized with a combination of 10 mg/kg of ketamine hydrochloride (CEVA Saúde Animal Ltda., Paulínia, SP, Brazil) and 0.5 mg/kg of xylazine hydrochloride (CEVA Saúde Animal Ltda., Paulínia, SP, Brazil) given as an intramuscular injection. If necessary, reapplication was done using half the dose of ketamine alone. Semen samples were collected by rectal probe electroejaculation (RPE), based on a previous report (Valle *et al*., 2004). Briefly, stimuli were applied for 2-3 sec, with an interval of 1-2 sec between each stimulus. Each series was composed of 10 to 30 stimuli, depending on the animal's response (i.e. penile erection). The voltage ranged from 2 to 8 V, with 0.5 V increases between each series of stimuli. The maximum duration of the procedure was 20 min, regardless of the success in semen collection. Each male was submitted to successive regular semen collections every other week and contributed with two to six semen samples. Samples were immediately diluted with Ringer lactate solution (Equiplex Indústria Farmacêutica Ltda., Goiânia, GO, Brazil) to a final volume of 500 µl. To assess semen pH, a sample was collected directly from the tip of the penis after electroejaculation, using a pH strip (Merck, Darmstadt, Germany). Semen volume was measured using a pipette (Gilson, Villiers-le-Bel, France). To determine sperm concentration, 20 µl of diluted semen was added to 180 µl of 10% formol saline solution (1:10 dilution). Sperm were counted using an improved Neubauer haemocytometer. Ten microliters of semen were placed on a glass slide under a cover slip to assess total and progressive motility using a light microscope. Total motility was the percentage of sperm with any type of tail movement, while progressive motility was the percentage of sperm with active movement, either linear or in large circles (World Health Organization - WHO, 2010). Plasma membrane integrity was assessed using an eosin-nigrosin commercial kit VitaScan® (Lucron Bioproducts, Ab Gennep, The Netherlands). Five microliters of semen were mixed with 5 µl of eosin and incubated for 30 sec before mixing with 5 µl of nigrosin and then a smear was prepared on a glass slide and allowed to air dry at 37°C. To determine acrosome integrity, both the commercial kit Spermac® and the Simple Staining Method (Pope *et al*., 1991) were used as a validation. For Spermac® staining, a smear was made with semen and allowed to air dry at 37°C for 10 min. The slide was plunged seven times in the fixative solution before immersion for 10 min and washed in clean tap water seven times. Excess water was dried with tissue paper without touching the smear. The slide was plunged seven times in solution A and immersed for 90 sec and washed as described before. Then it was plunged in solution B seven times and immersed for one minute, washed and plunged in solution C seven times and immersed for 80 sec and then washed one last time. Then it was allowed to air dry at 37°C. For the simple staining method 5 µl of semen was mixed with 5 µl of the stain and incubated for 120 sec in the dark before preparing a smear on a glass slide. The smear was allowed to air dry at 37°C. To investigate mitochondrial activity, 10 µl of semen were mixed with 10 µl of DAB (Hrudka, 1987) in an amber centrifuge tube and incubated for one hour in a water bath at 37°C in the dark. After incubation a smear was prepared on a glass slide and allowed to air dry at room temperature (25°C) in the dark. When the slide was dry it was immersed in a 10% formol solution for 10 min and then air dried at room temperature. The whole procedure was done in very low light conditions. Mitochondrial activity was classified in four classes according to Hrudka (1987): Class I - almost all mitochondria are active; Class II - more than 50% of mitochondria are active; Class III - less than 50% of mitochondria are active; Class IV - none of the mitochondria are active. With the exception of mitochondrial activity, all the slides were read using a light microscope (Nikon Corporation, Tokyo, Japan) and a 100X objective lens, under oil immersion. For the mitochondrial activity a phase contrast microscope (Nikon Corporation, Tokyo, Japan) was used with the same objective lens.

Statistics

Data were analyzed using the statistical software SAS System for Windows (SAS Institute Inc, Cary, NC, USA, 2000). The Analysis of Variance (ANOVA) procedure and the least significant difference (LSD) means procedure were done to compare means

between males and collections. To validate the Simple Staining Method and Spermac® stain for acrosome integrity, Pearson correlation coefficient was applied. Results were considered significant when $P \leq 0.05$.

Results

Testicular measures are shown in Table 1. There was inter- and intra-individual variation in testicular volume. When the larger testicle of each male was taken as reference, the contralateral testicle was 6 to 22% smaller. Total testicular volume (left testis volume + right testis volume) also differed between males. There was no correlation between testicular volume and number of sperm/ejaculate (r = -0.38 and P = 0.447). Male AAF had the largest testicle and a mean total number of sperm/ejaculate of 3,906,300, while male BAB, had the smallest testicle and a mean total number of sperm/ejaculate of 44,696,552. The male with the largest number of sperm/ejaculate (BAC) had a testicular volume of 10.19 cm^3, which is less than the mean (13.18 cm^3) for the studied animals.

Table 1. Testicular morphometry of six captive black howler monkeys (*Alouatta caraya*) from the National Primate Center, Ananindeua, PA, Brazil.

Male	Age (years)	Right Testicle (cm)[a]				Left Testicle (cm)[a]				TV (cm^3)[b]	SP(cm)[c]
		L	W	H	V (cm^3)	L	W	H	V (cm^3)		
BAC	6[d]	2.58	1.68	1.56	4.81	2.59	1.67	1.75	5.38	10.19	11.00
BAB	5	2.30	1.58	1.39	3.58	2.24	1.54	1.37	3.35	6.94	10.00
AAF	16[d]	2.79	2.33	2.10	9.64	3.24	2.40	2.25	12.43	22.08	9.00
ABJ	16	2.56	1.78	1.58	5.12	2.51	1.48	1.63	4.32	9.45	10.00
AFZ	16[d]	2.73	1.84	1.99	7.13	2.98	1.99	1.84	7.77	14.90	11.50
BAA	8	2.22	2.09	2.13	7.03	2.64	2.13	2.12	8.48	15.51	11.00

[a]L = length; W = width; H = height; V = volume; [b]TV = total volume; [c]SP = scrotal perimeter; [d]wild born – estimated ages.

A total of 26 ejaculates were collected from six males. Descriptive statistics - mean ± standard error and range (minimum - maximum) for all the parameters analyzed are shown in Table 2. Although there are three males considered of old age (16 years old) there was no statistical difference ($P > 0.05$) between semen characteristics of the young males (5-8 years old) and the old males. Therefore, all the animals were considered as one group for statistical analyses.

Table 2. Semen characteristics of captive black howler monkeys (*Alouatta caraya*) from the National Primate Center, Ananindeua, PA, Brazil.

Parameter	Ejaculates (n)	Mean ± SEM	Range
Volume (µl)	26	86.80 ± 10.40	20.00 - 218.00
pH	26	7.45 ± 0.06	6.60 - 7.80
Concentration (10^6 sperm/ml)	26	726.00 ± 129.00	52.30 - 2489.00
Total motility (%)	26	60.00 ± 5.57	5.00 - 90.00
Progressive motility (%)	26	47.15 ± 5.47	0.00 - 80.00
Intact plasma membrane (%)	26	47.38 ± 3.21	19.00 - 82.00
Intact acrosome (Spermac; %)	26	56.15 ± 2.92	26.00 - 78.00
Intact acrosome (Simple Stain; %)	26	58.96 ± 3.48	18.00 - 83.00
MA[a] Class I (%)	24	7.50 ± 1.53	0.00 - 28.00
MA[a] Class II (%)	24	51.17 ± 4.23	12.00 - 82.00
MA[a] Class III (%)	24	31.54 ± 3.54	8.00 - 71.00
MA[a] Class IV (%)	24	9.79 ± 2.33	0.00 - 50.00

[a]MA = mitochondrial activity

Table 3 contains results for ANOVA and means comparison for each male (n = 6). According to ANOVA, there were significant differences between males only for semen concentration (P = 0.0009) and plasma membrane integrity (P = 0.0271). No other parameter differed significantly between males.

Regarding the validation of the acrosome staining techniques, there was a very high correlation (R = 0.97; P < 0.0001) between the Simple Staining Method and the Spermac® stain.

Table 3. Semen characteristics (mean ± standard error) of six captive black howler monkeys (*Alouatta caraya*) from the National Primate Center, Ananindeua, PA, Brazil and results of ANOVA and LSD tests.

Parameter	Animal						ANOVA		
	BAC	BAB	AAF	ABJ	AFZ	BAA	CV	F	P
Ejaculates (n)	6	5	2	4	5	4			
Volume (µl)	110.00 ± 26.70	65.40 ± 6.62	22.50 ± 2.50	122.25 ± 31.16	87.60 ± 14.07	74.25 ± 32.33	57.88	1.54	0.2218
pH	7.60 ± 0.07	7.48 ± 0.10	7.05 ± 0.35	7.55 ± 0.05	7.40 ± 0.21	7.38 ± 0.09	3.10	1.33	0.2932
Concentration (x10^6 sperm/ml)*	1533.62^{a} ± 325.42	682.83^{b} ± 152.95	164.69^{b} ± 80.31	203.76^{b} ± 87.61	842.82^{b} ± 106.78	223.81^{b} ± 79.51	62.03	6.63	0.0009
Total motility (%)	60.83 ± 12.94	69.00 ± 16.16	30.00 ± 20.00	60.00 ± 13.54	68.00 ± 6.63	52.50 ± 16.52	49.15	0.63	0.6767
Progressive motility (%)	48.50 ± 12.37	58.00 ± 14.97	10.00 ± 10.00	50.00 ± 13.54	56.00 ± 6.00	36.25 ± 15.73	58.38	1.12	0.3796
Intact plasma membrane (%)*	44.67^{ab} ± 4.76	56.60^{a} ± 8.99	36.50^{ab} ± 0.50	27.00^{b} ± 4.83	54.60^{a} ± 6.90	56.75^{a} ± 3.75	28.70	3.22	0.0271
Intact acrosome spermac (%)	66.83 ± 4.51	56.00 ± 7.56	45.00 ± 10.00	46.00 ± 10.09	58.80 ± 6.17	52.75 ± 3.90	25.55	1.39	0.2716
Intact acrosome Simple Stain (%)	68.67 ± 6.02	56.60 ± 10.71	40.50 ± 8.50	50.50 ± 11.21	66.20 ± 5.69	56.00 ± 5.15	29.37	1.24	0.3281
MA[a] Class I (%)	7.33 ± 4.52	4.75 ± 1.93	8.00 ± 3.00	15.00 ± 4.62	3.60 ± 1.69	9.50 ± 3.50	99.79	1.04	0.4238
MA[a] Class II (%)	49.17 ± 9.94	57.75 ± 10.59	50.00 ± 6.00	58.33 ± 8.74	34.40 ± 11.19	63.75 ± 3.33	39.84	1.15	0.3699
MA[a] Class III (%)	31.17 ± 7.13	33.25 ± 11.14	33.50 ± 10.50	18.33 ± 3.33	44.20 ± 9.62	23.50 ± 3.48	54.39	1.09	0.3997
MA[a] Class IV (%)	12.33 ± 5.23	4.25 ± 1.44	8.50 ± 1.50	8.33 ± 1.76	17.80 ± 8.45	3.25 ± 1.44	115.96	1.03	0.4305

[a]MA = mitochondrial activity; *Means followed by the same letters do not differ statistically.

Comparisons between subsequent collections are shown in Fig. 1 through 7. There was no difference between collections for the volume, pH, and plasma membrane integrity parameters. Sperm concentration was significantly higher in the fifth collection when compared to the first collection. Total and progressive motility were significantly higher in the fourth collection when compared to the first collection. Acrosome integrity was significantly higher in the third collection when compared to the first and second collections and in the fifth collection when compared to the first three collections. Mitochondrial activity Class I was significantly higher in the first collection than in all the other subsequent collections. Mitochondrial activity class II was significantly higher in the third collection when compared to the fourth collection and was similar in all the other collections.

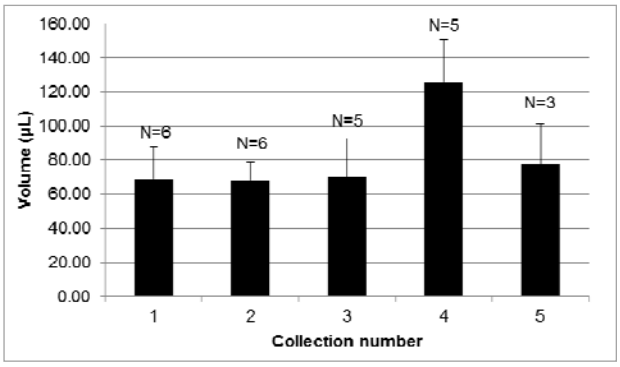

Figure 1. Mean semen volume for each of the five subsequent sperm collections from *Alouatta caraya* (n = 3-6 animals)

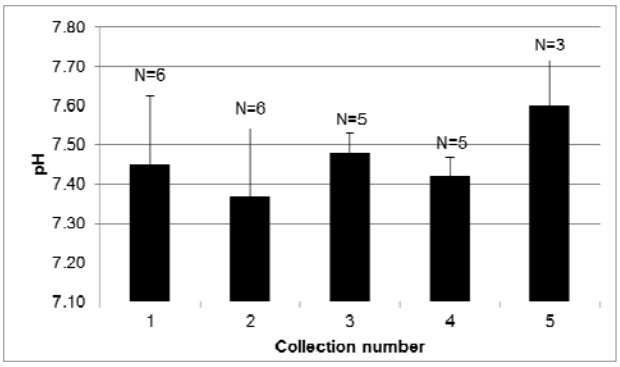

Figure 2. Mean semen pH for each of the five subsequent sperm collections from *Alouatta caraya* (n = 3-6 animals).

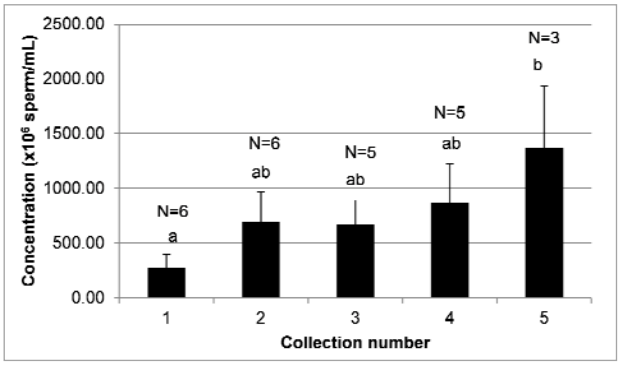

Figure 3. Mean sperm concentration for each of the five subsequent sperm collections from *Alouatta caraya* (n = 3-6 animals). Similar bars with the same letter above do not differ statistically according to LSD tests.

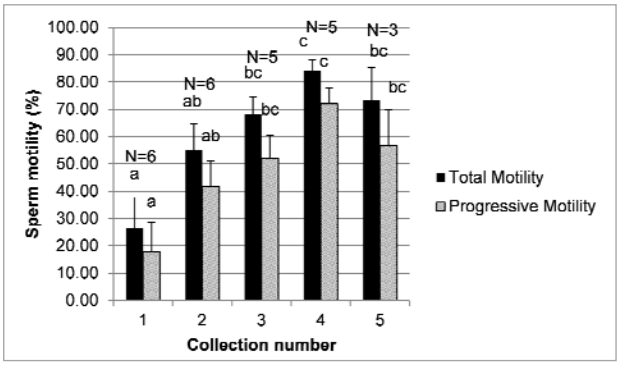

Figure 4. Mean percentage of total and progressive sperm motility for each of the five subsequent sperm collections from *Alouatta caraya* (n = 3-6 animals). Similar bars with the same letter above do not differ statistically according to LSD tests.

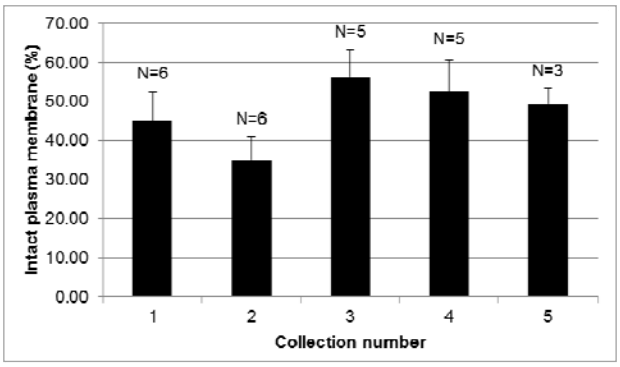

Figure 5. Mean percentage of spermatozoa with intact plasma membrane for each of the five subsequent sperm collections from *Alouatta caraya* (n = 3-6 animals).

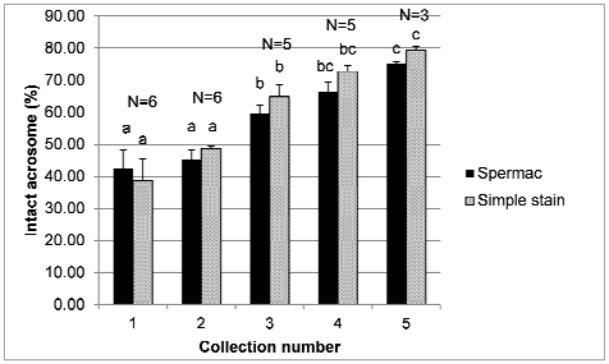

Figure 6. Mean percentage of spermatozoa with intact acrosome for each of the five subsequent sperm collections from *Alouatta caraya* (n = 3-6 animals), using two different staining methods. Similar bars with the same letter above do not differ statistically according to LSD tests.

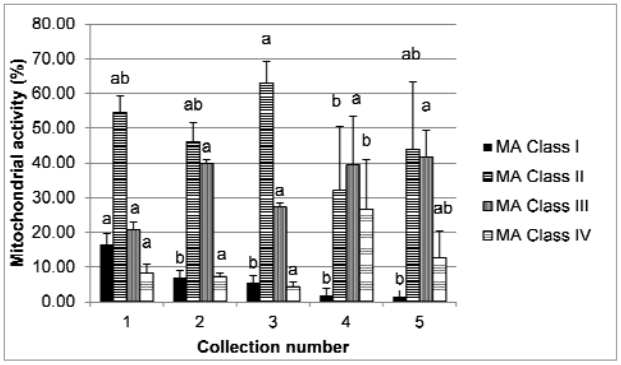

Figure 7. Mean percentage of spermatozoa classified according to mitochondrial activity classes for each of the five subsequent sperm collections from *Alouatta caraya* (n = 3-6 animals). MA = mitochondrial activity. Similar bars with the same letter above do not differ statistically according to LSD tests.

Discussion

Total testicular volume varied from 6.94 to 22.08 cm^3, with a mean of 13.18 cm^3. This mean was smaller than the one reported by Moreland *et al*. (2001) for *Alouatta caraya* (16.70 cm^3). These authors used another formula, which assumes that the width and height of the testicles are alike, when they are not. Moreover, when we applied the same formula used by those authors, our mean (10.11 cm^3) was even smaller than the mean obtained by Lambert's formula. Moreland's study was done year-round and animals were maintained both in indoor enclosures and outdoor enclosures, in South Carolina, USA. Although testicular measures did not differ statistically during the year, the authors observed a peak in June (summer). Our data were collected during the spring, which could possibly explain the smaller mean. Additionally, there may be genetic differences between populations from different origins. In this study testicular volume was not associated with sperm production, as was observed in an interspecific analysis which included 25 primate species, including Neotropical primates, Old World primates and humans (Moller, 1988). This may be due to the small number of animals used or differences in testicular anatomy in this species - for example, larger testes may mean more parenchyma rather than more seminiferous tissue. Further investigation is necessary to determine if this is a characteristic of this species or just a consequence of the small number of animals used.

Ejaculate volume varied between 20 and 218 µl, with a mean of 86.77 µl. These values were similar to those found in earlier studies with *Alouatta caraya*, which varied between 10 and 203 µl (Moreland *et al*., 2001; Valle *et al*., 2004). Semen pH ranged from 6.6 to 7.8, with a mean of 7.45 ± 0.06, much lower than the means found in previous studies which were 8.9 ± 0.1 (Moreland *et al*., 2001) and 8.1 ± 0.5 (Valle *et al*., 2004). According to WHO (2010), semen pH in humans is determined by the balance between the secretions of the accessory glands, especially the seminal vesicles (alkaline pH) and the prostate (acid pH). Moreover, semen pH tends to increase over time, due to the loss of CO_2, which reduces buffering capacity. Therefore, differences in pH may be due to differences in the stimulation of the accessory glands, according to the size and/or position of the electrodes and to differences in the time of pH measurement. Additionally, as suggested by Valle *et al*. (2004), nutritional or environmental factors could affect acid-base balance with further changes in semen pH.

Sperm concentration was between 52.29 x 10^6 and 2489.36 x 10^6 sperm/ml, with a mean of 725.75 ± 129 x 10^6 sperm/ml. These values are within those found by other authors, which ranged from 7 x 10^6 to 5400 x 10^6 sperm/ml (Moreland *et al*., 2001; Valle *et al*., 2004).

Total motility was between 5 and 90%, with a mean of 60 ± 5.57%, values lower than those found in previous research, which ranged from 30 to 95% (Moreland *et al*., 2001; Valle *et al*., 2004). Progressive motility was between 0 and 80%, with a mean of 47.15 ± 5.47%. The other studies used a different classification method to evaluate progressive motility, making it impossible to compare results.

As highlighted in the introduction, no reports were found on acrosome integrity and mitochondrial activity for Black-and-gold howler's sperm. The former is very important since the acrosome contains enzymes involved in the fertilization process (Garner and Hafez, 2004) and, thus, the percentage of spermatozoa with intact acrosome is directly associated with semen fertility. Acrosome integrity varied between 24 and 83%, with means of 56.15 ± 2.92% (Simple Stain) and 58.96 ± 3.48% (Spermac® stain). These means are similar to those found for *Cebus apella* (61.8 ± 19.7% and 67.4 ± 16.3%; Paz *et al*., 2006a, b) and for *Callimico goeldii* (61 ± 6% and 65 ± 9%; Arakaki *et al*., 2011). However, it is lower than the 84.13 ± 2% and 85.04 ± 3.93% means found for *Callithrix jacchus* in

Brazil and Germany, respectively (Valle, 2007), and 78.9 ± 2.7% for *C. penicillata* (Massaroto *et al*., 2010). High mitochondrial activity (classes I and II) was found in 58.67% of the spermatozoa. This value is lower than that obtained for *Callithrix penicillata* in which 78.35 ± 3.4% were classified as class I (Massaroto *et al*., 2010). In addition to species related differences, the lower semen quality regarding acrosome integrity and mitochondrial activity may be due to the collection method, whereas in the present study and in the studies with *Cebus apella* and *Callimico goeldii*, semen was collected by RPE, while in the studies with *Callithrix jacchus* and *C. penicillata* semen was collected by penile vibrostimulation (PVS). Studies with *Saimiri boliviensis* (Yeoman *et al*., 1997, 1998) and *Callithrix jacchus* (Schneiders *et al*., 2004) have demonstrated that PVS yields better quality semen than RPE.

Overall semen quality increased with each subsequent collection. This was specially noted for total and progressive motility and acrosome integrity. A study with bull spermatozoa showed that semen from animals on sexual rest had poor quality, which increased over time with subsequent weekly collections (Wells *et al*., 1970). These authors gave special attention to the acrosome and demonstrated that the percentage of abnormal acrosome decreased significantly from the first collection to the sixth collection. Results obtained in our study corroborate their findings. Improvement in semen quality in successive regular collections was also observed in the Indo-Pacific bottlenose dolphin (*Tursiops aduncus*; Yuen *et al*., 2009) and in the Sumatran rhinoceros (*Dicerorhinus sumatrensis*; Agil *et al*., 2004). Yuen *et al*. (2009) suggested that the lower motility and acrosome integrity of the initial ejaculates may be due to the presence of aged spermatozoa from previous ejaculations or to differences in seminal plasma composition between successive collections. In the present study males that were paired with females were separated from them 24 h prior to semen collection, to avoid copulation during this period, but there was no control of when the last copulation occurred before they were separated. Therefore, it is possible that there were old spermatozoa stored in the epididymis that were eliminated during the initial ejaculations and replaced by new spermatozoa. Although semen collection was always done by the same person, there may be differences in plasma composition between collections due to differences in the positioning of the probe. This may happen because of anatomical variations between individuals (i.e. size and position of the prostate), presence of feces in the rectum, and slight differences in the angle of the probe in relation to the rectum wall. Nevertheless, it is unlikely that this would explain the improvement in semen quality observed in this study, since it was consistent across collections.

Mitochondrial activity class I significantly decreased from the first collection to the second collection and continued to decrease in subsequent collections, although not significantly. In other words, as semen quality increased, mitochondrial activity decreased. The same pattern was observed in a cryopreservation study done with the same semen samples (Carvalho, 2012). As semen quality decreased (fresh versus cryopreserved semen), mitochondrial activity increased. A possible explanation for this is that spermatozoa from *Alouatta caraya* may offer some resistance to the penetration of DAB, as described for swine semen (Hrudka, 1987). Poor quality semen may allow penetration of the stain through the non-intact membrane, whereas high quality semen offers resistance. This might be overcome by increasing incubation time with the stain, as observed for swine spermatozoa (Hrudka, 1987).

In conclusion, semen quality tends to improve with successive regular collections, a fact that highlights the importance of analyzing various semen samples from each male before its use in ART programs. Moreover, we were able to validate both techniques for acrosome staining, whereas the technique to assess mitochondrial activity needs further investigation to improve its application for this species. This research brought novel information on semen characteristics of *Alouatta caraya* which will aid further studies on the application of ARTs in this and other primate species.

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