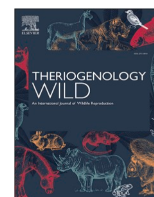
Theriogenology Wild 5 (2024) 100098

Contents lists available at ScienceDirect

Theriogenology Wild

journal homepage: www.journals.elsevier.com/theriogenology-wild



# Influence of age and weight on seminal parameters of golden-headed lion tamarin (*Leontopithecus chrysomelas*) in *ex situ* conditions and potential use of seminal coagulum for molecular procedures

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A R T I C L E I N F O

*Keywords:*
Reproductive biotechniques
*ex situ* conservation
RNA Extraction

A B S T R A C T

The golden-headed lion tamarin (*Leontopithecus chrysomelas*) is an endangered primate endemic to the Atlantic Forest. Conservation efforts for the species involve applying reproductive biotechniques to preserve genetic resources and ensure the management of populations in both *ex situ* and *in situ* conditions. This study aims to initiate investigations into seminal and molecular factors influencing the reproductive potential of sexually mature males. Semen was collected using the penile vibrostimulation technique, and seminal parameters were assessed in two groups: the 'Old' group (average age 11.6 years; n=6) and the 'Young' group (average age 4.8 years; n=6). ANOVA results indicated age-related influences on plasma membrane integrity (p=0.049), acrosomal integrity (p=0.009), and DAB IV (p=0.026) for both groups. Linear regression revealed significant correlations between seminal parameters and age (plasma membrane integrity (p=0.021), acrosomal integrity (p=0.05), and DAB III (p=0.024)), alongside animal weight (plasma membrane integrity (p=0.010), acrosomal integrity (p=0.009), DAB III (p=0.33), and DAB IV (p=0.066)). In an effort to advance reproductive techniques and sperm selection, a protocol utilizing a discontinuous Percoll gradient was employed. Despite its effectiveness in isolating gametes, there were no significant gains in the reevaluated parameters post-selection, necessitating adjustments in the methodology. While semen cryopreservation is common in wild species, challenges arise due to seminal coagulum in many neotropical primate ejaculates, hindering gamete use in reproductive procedures. Given the precious nature of and the considerable effort involved in collecting semen from these animals, it would be desirable to maximize the sample's utility. The liquid fraction could be applied in reproductive biotechniques, while the spermatozoa contained in the clot could be utilized as a non-invasive approach for molecular evaluation of these gametes. This study established a protocol for RNA extraction from sperm retained in the seminal coagulum, highlighting its genetic richness often discarded post-processing. In summary, our study emphasizes the importance of early cryopreservation of semen to safeguard the reproductive potential of *L. chrysomelas*. Additionally, we propose further exploration of RNA quantity in gametes as a non-invasive tool for inferring male fertility, given the pivotal role of sperm RNA transcripts in regulating the activation of the female gamete and gene expression during early embryo development.

## Introduction

The golden-headed lion tamarin (*Leontopithecus chrysomelas*) is a primate belonging to the Callitrichidae family, endemic to the Atlantic Forest [1]. Similar to other species in the *Leontopithecus* genus, the golden-headed lion tamarin is categorized as threatened [2], with the

*Abbreviations:* PVS, penile vibrostimulation; DAB, 3,3′- diaminobenzidine; ICC, Intraclass Correlation Coefficient.

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https://doi.org/10.1016/j.therwi.2024.100098
Received 15 February 2024; Received in revised form 11 June 2024; Accepted 12 June 2024
Available online 13 June 2024
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primary cause of its population decline attributed to the extensive history of biome fragmentation and exploitation [1,3]. *Ex situ* management of the species began in the 1970s and has since demonstrated a successful track record in captive breeding programs. Studies even indicate a higher reproductive rate among individuals born under human care, attributed to the mitigation of environmental unpredictabilities and resource limitations, factors that often restrict reproductive potential in natural environments [4–6].

One of the main challenges in maintaining *ex situ* populations is the preservation of genetic variability, as genetically healthy populations can mitigate inbreeding depression and enable better adaptation to this environment [7]. In this context, technologies have been developed/adapted to facilitate genetic management among populations, and the use of male gametes appears promising, due to their ease of acquisition and other characteristics inherent to male reproduction. It also allows overcoming challenges of sexual incompatibility, such as male aggression or preferential male selection, and can preserve the reproductive potential of an individual even after death through long-term storage methodologies [5]. The golden-headed lion tamarin has emerged as a potential model within the *Leontopithecus* genus for the application of reproductive biotechnologies [8,9], underscoring the imperative for further research into the reproductive biology of this species. Such studies can provide valuable insights to reproductive management and the validation of species-specific protocols, given that reproductive mechanisms may not be universally conserved across species [5,10,11]. Interindividual variations are also crucial in the application of reproductive techniques, with a comprehensive understanding of individuals' reproductive potential and the factors that may influence it being essential [12].

Semen parameters are essential factors to be considered regarding viability, sperm function, and fertilization success [13]. Traditionally, semen is assessed for ejaculate volume, motility, sperm concentration, and morphology. While these characteristics alone may not provide a comprehensive evaluation of sperm quality [14], sperm motility plays a significant role, correlating positively with fertility and negatively with low heterozygosity and high rates of inbreeding [15,16]. Other tests of sperm viability are also used to evaluate seminal profiles, such as the integrity of plasma and acrosomal membranes and determination of mitochondrial function [17–19], and they are already validated for neotropical primates, such as *Callithrix jacchus* [20] and *Leontopithecus chrysomelas* [9]. However, accessibility to these seminal parameters in neotropical primates is limited, as their semen has the characteristic of coagulating shortly after or during ejaculation, forming less dense coagula or even compact plugs [21], except for *Alouatta caraya, A. guariba* [22,23], *A. palliata, and A. pigra* (M. A. Hirst, personal communication), which semen do not form coagulum. Due to this peculiarity, even with the use of extenders, for almost all studied species the seminal coagulum is seldom fully liquefied, affecting the availability of gametes with fertilization potential, posing a major limitation to reproductive biotechnologies [8,24,25], and the remaining coagulum from semen dilution is often discarded [26]. As the semen collection procedure in tamarins involves special care to minimize animal stress, the ideal scenario would be to maximize sample utilization, ensuring that the ejaculate is used in its entirety, even for different purposes. such as artificial insemination, sperm analysis, or cryopreservation.

Despite some isolated studies investigating the influence of age on seminal parameters in Old World primates [27] and the proteomic analysis of testicular aging in marmosets [28], reports on this influence on the reproductive potential of *ex situ* Platyrrhini primate males are still incipient. It is noteworthy that body weight is likely to play a role in the reproduction of these animals, as seasonal fat gain seems to be associated with sperm production in squirrel monkeys [29,30]. In *Callithrix jacchus*, a positive correlation was found between male weight and the incidence of sperm morphological abnormalities [31]. Thus, examining reproductive and molecular parameters that may influence reproductive potential can contribute practical knowledge to the species' reproductive management. In this study, we investigate the influence of age and weight on seminal parameters in young and old individuals of the golden-headed lion tamarins, housed in captivity. Additionally, we tested sperm selection in a Percoll® gradient, aiming to develop species-specific protocols to contribute to the preservation of genetic resources in Platyrrhini primates. As the liquefied semen fraction would be utilized for reproductive biotechnologies, we aimed to maximize the sample's utility for molecular studies by using the remaining gametes from coagulum. Consequently, for molecular parameters, we established a protocol for RNA extraction from the sperm fraction of the remaining seminal coagulum to eliminate somatic cells in the sample. The prospect of applying reproductive biotechnologies and potential molecular studies can assist in the reproductive management decisions of the species in *ex situ* conditions. Additionally, it can contribute to the breeding programs of closely related species that do not have the same history of reproduction under human care, serving as targets for future reproductive interventions.

## Materials and methods

### *Animals*

Twelve sexually mature males of golden-headed lion tamarin (4–14 years old) housed at the Fundação Parque Zoológico de São Paulo were used in this study. It is worth noting that sexual maturity in tamarins (*Saguinus* and *Leontopithecus*) is described by Hoage [32] as occurring from 24 months onward. The males were divided into two groups: the "Old" group, consisting of animals aged 8–14 years, and the "Young" group, with animals between 4 and 5 years old. The animals were kept in separate family groups in semi-open enclosures, providing refuge points and exposure to local climate changes. The diet, formulated by the Animal Feeding Sector of the institution, was balanced according to the species' nutritional needs, and water was available *ad libitum*. All physical restraint of the animals was performed by a trained professional from the Veterinary Sector. The procedures were conducted with the approval of the Ethics Committee for Animal Use at the Federal University of São Carlos (CEUA/UFSCar N ° 045/2014) and in compliance with Brazilian legal requirements (SISBio No. 46715–1). This study adheres to the American Society of Primatologists (ASP) Principles for the Ethical Treatment of Non-Human Primates.

### *Semen collection and processing*

The collections were performed in 2015 (June, August, and December), based on the logistics of the zoo's staff. Sampling took place in the morning, with most animals having three different samples collected on separate days, with at least one week between each collection.

The penile vibrostimulation (PVS) technique was employed for semen collection, following the procedure outlined in Arakaki et al. [8]. A hand-held personal vibrator (FertiCare® personal; Multicept ApS, Rungsted, Denmark) used for the stimulus was attached to a small cylindrical glass tube with rounded edges, simulating an artificial vagina, where the semen was collected. The collection involved perianal region stimulation and the insertion of the penis into the glass tube (Fig. 1), maintaining a fixed amplitude (1 mm) and frequency that was gradually increased based on each animal's response (70–80 Hz). The procedure was halted if the animal did not ejaculate within the prescribed 20-minute stimulation. After this interval, the procedure was concluded and considered unsuccessful. To minimize stress, food items such as insect larvae and fruits were offered to the animals during the stimulus intervals.

Immediately after ejaculation, the seminal pH was measured using a pH strip (Merck®, Darmstadt, Germany) directly on the animal's glans. In addition to pH measurement, the samples were monitored for urine contamination based on volume and coloration. It is noted that animals

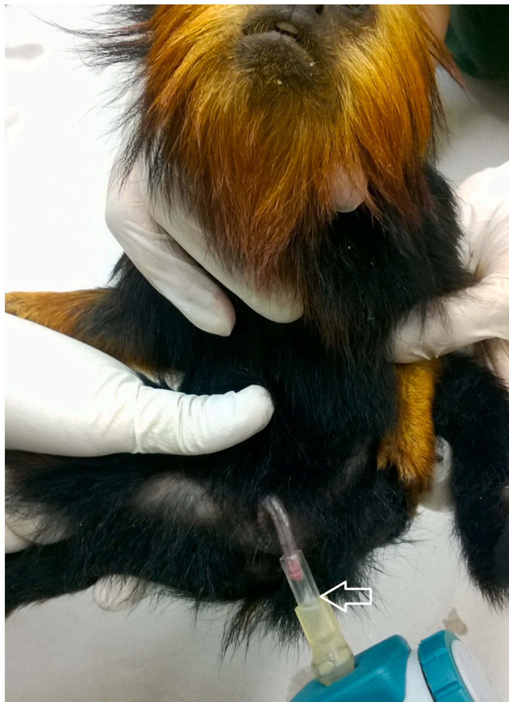

**Fig. 1.** Semen collection by penile vibrostimulation technique. The arrow indicates the seminal coagulum formation just after ejaculation.

rarely urinate during this procedure, with none recorded in this study. The collected semen was then diluted into Biggers-Whitten-Whittinghan (BWW) medium at 37°C, for 30 minutes. After this period, the ejaculate volume was quantified by weighing the tube containing the diluted semen using a precision scale (AUX220; Shimadzu®, Tokyo, Japan), and then the tubes weight and the amount of extender used were subtracted. A small portion of the liquid fraction (extender plus sperm cells) was used for seminal parameters measurements. The remaining volume was utilized to select sperm through discontinuous Percoll® (*GE Healthcare Bioscience* - Uppsala, Sweden) gradients of 40/80 %, as modified from the protocol described by Prakash et al. [33]. Briefly, the liquid fraction was quantified and added above the gradient, placed in a 1.5 mL microtube, and maintained at 37 °C. Following centrifugation (500xG at 37 °C, 20 min), the upper layer was removed, and the pellet was resuspended in a 1:2 ratio in BWW medium. Once again, a small portion of the post-selection sperm was used for seminal parameter evaluation. Both the remaining samples from sperm selection and the residual seminal coagulum were separately stored in *RNA holder* (*BioAgency*, São Paulo, Brazil) and kept at −80°C for molecular procedures.

*Semen parameters evaluations*

The semen parameters analyzed here were measured in both sample groups, before and after semen selection using discontinuous Percoll® gradients.

*Sperm motility and concentration*

Ten microliters of the liquid fraction placed in a 37°C glass slide was used to analyze total and forward progressive sperm motility. Sperm concentration was determined in a hemocytometer chamber by diluting samples in 1:10 or 1:20 in 10 % formol saline solution at 37°C.

*Plasma membrane and acrosome integrity*

The assessment of plasma membrane integrity followed an Eosin (1 %)-Nigrosine (10 %) staining procedure [9,20]. After a 30-second incubation at 37°C, the smear was evaluated at 1000x magnification, and plasma membrane integrity was measured by the eosin/nigrosine dye penetration. Acrosome integrity was assessed using the Fast-Green/Rose Bengal staining protocol described by Pope et al. [34] and validated for *Leontopithecus chrysomelas* [9]. We counted 200 cells at 100x magnification, and the results were expressed as the percentage of intact cells, determined by acrosome staining.

*Mitochondrial activity and sperm morphology*

The 3,3′-diaminobenzidine (DAB) staining [35] was used to assess mitochondrial activity, validated for *Leontopithecus chrysomelas* [9]. A semen sample (10 μL) was incubated with 20 μL DAB at 37°C for one hour. Ten μL were smeared and air-dried in the dark, followed by fixation in 10 % formaldehyde for 10 minutes. Two hundred cells were examined using a phase-contrast optical microscope (1000x magnification) and classified into four different categories: DAB I – total mitochondria activity (100 % of midpiece stained); DAB II – the majority of mitochondria activity (more than 50 % of the midpiece stained); DAB III – the minority of mitochondria activity (less than 50 % of the midpiece stained); and DAB IV - mitochondrial inactivity (no staining in the midpiece).

Sperm morphology analyses were assessed using wet-mount semen fixed in formol saline solution. There were 10 μL of sample from a dilution in 1:10 or 1:20 in 10 % formol saline solution, and two hundred cells were assessed using a phase-contrast microscope (Nikon® E200, Tokyo, Japan - 1000x magnification), following Blom *et al.* [36], distinguishing normal sperm morphology from abnormal morphology (classified into major or minor defects).

*Molecular procedures*

*RNA extraction*

Three different semen samples from each animal were concentrated into two distinct pools: (1) Liquid Fraction Pool, referring to the pellet resulting from discontinuous gradient selection of Percoll®, and (2) Seminal Coagulum Pool which represents the portion of the ejaculate that did not liquefy after incubation in BWW medium. Both pools underwent centrifugation at 10000xG for 5 minutes at 4°C, and the pellets were recovered. Samples were mechanically macerated, and for the seminal coagulum pool, a lancet was used to cut them into pieces. Before the sperm RNA extraction, a pre-lysis of the remaining somatic cells was performed in both pools by exposing the sample to a hypotonic solution (0.5 % Triton-X), adapted from Rauber [37]. After 10 minutes on ice, the tubes were centrifuged at 5000xG for 5 minutes at 4°C. The supernatant was discarded, and the pellet was washed with 1x PBS prepared from a stock solution (250 mM $Na_2HPO_4$; 1.25 mM NaCl; 250 mM $KH_2PO_4$; 1.25 mM KCl).

Sperm RNA extraction was performed using the Phenol/Chloroform protocol [38]. Briefly, the resulting pellet was resuspended in 1 mL of a solution containing TRIzol® (Invitrogen) and β-mercaptoethanol (7 μL for each milliliter of TRIzol®). The contents were passed three times through the 30 G needle, vortexed for 30 seconds, and incubated on ice for 5 min. The samples were centrifuged at 12000xG for 10 minutes at 4 °C. The volume was transferred to a new tube, and 300 μL of chloroform solution and isoamyl alcohol (24: 1) was added. Samples were vortexed and incubated on ice for 5 minutes, then centrifuged at 12000xG for 15 minutes at 4 °C. The organic phase was transferred to a new microtube containing 1 vol of 100 % isopropanol, 1 μl azure glycogen, and 0.1 vol of 7.5 M ammonium acetate. Microtubes were incubated for 30 minutes on ice and centrifuged at 14000xG for 30 minutes at 4 °C. The supernatant was carefully removed with a pipette, and the pellet was washed with 500 μl of 80 % ethanol. After drying, the pellets were resuspended in 20 μl of water with Diethylpyrocarbonate - DEPC (Invitrogen) and stored at −80 °C.

*RNA quantification and analysis*

Each sample was quantified for detection of large and small RNAs

molecules using the Qubit® RNA HS Assay Kit (Life Technologies, USA) and the Qubit® microRNA Assay Kit (Life Technologies, USA), respectively. Total RNA profiles extracted from the liquid fraction and seminal coagulum of golden-headed lion tamarin were evaluated with Bioanalyzer (Agilent Technologies, USA), an electrophoresis platform for qualitative and quantitative RNA analysis using the RNA 6000 Pico kit (Agilent Technologies, USA), for viewing on samples with very low RNA concentrations.

### *Statistical analysis*

Data distribution was assessed using GraphPad Prism (version 6.01, San Diego, USA) with the Shapiro-Wilk normality test. Parameters that deviated from the assumption of normality ($p>0.05$) were transformed into base 10 and square root logarithms and re-evaluated for asymmetry (between −2 and 2) and kurtosis (between −3 and 3). To address extreme values, any value outside three standard deviations from the mean at both ends of the distribution was considered an outlier and were removed.

The consistency of replicates for semen collection was tested using two complementary methods: (1) Intraclass Correlation Coefficient (ICC), analyzed with the MedCalc program (version 16.2.1, Ostend, Belgium), and (2) ANOVA for repeated measurements, performed with GraphPad Prism software (version 6.01, San Diego, USA). For data with low consistency, the Mauchly sphericity test was conducted using SPSS program (version 23, London, England).

The difference between the two sampled groups, “Old" and "Young", was assessed using the *Student’s* t-test with Sidak-Bonferroni correction ($\alpha = 0.05$) for multiple comparisons on the GraphPad Prism (version 6.01, San Diego, USA). The repeated measures ANOVA test was employed to compare the seminal parameters evaluated before and after discontinuous Percoll® gradient selection.

Multiple linear regression was used to examine the association and type of influence of weight and age variables on evaluated seminal parameters: stimulation time, pH, ejaculate volume, concentration, motility, morphology, plasma membrane and acrosome integrity, and mitochondrial activity.

## Results

### *Individual and group description data*

Table 1 displays data on semen collection aspects for all animals involved in this study. The animals are categorized into two groups: the ’Old’ group, consisting of animals aged 8–14 years old, with an average body weight of 711 g, and the ’Young’ group, comprising animals between 4 and 5 years old, with a significantly lower mean body weight of 687 g ($p = 0.048$). Out of 36 attempts of semen collection in golden-headed lion tamarins using the penile vibrostimulation technique, there was a success rate of 86.1 %. Failure occurred in two collection attempts on a single individual in the ’Old’ group. When comparing between groups, the ’Young’ animals had a 94 % success rate, while the ’Old’ animals achieved success in 77.7 % of the 18 attempts for both groups. The differences in ejaculation latency (time), semen pH, and volume parameters observed between the groups were not statistically significant.

### *Semen parameters evaluations and age and weight influences*

Table 2 displays the mean values for the evaluations of the three semen collection parameters before semen selection. As observed, the differences found between groups were not significant for sperm concentration, total motility, and progressive motility parameters. However, the ‘Old’ group exhibited significantly lower plasma membrane integrity and acrosome integrity than animals from ‘Young’ group ($p=0.049$; $p=0.009$, respectively). For details on seminal parameters, refer to the Appendices (Tables A1-A5).

Among the total sperm evaluated for mitochondrial activity, only DAB IV showed significant differences between the two groups ($p = 0.026$), indicating that the "Old" animals had a significantly higher number of inactive mitochondria in sperm. Sperm morphology revealed no significant differences between the groups. In general, major defects found were a strongly folded tail (15.73 %) and intermediate part defects (3.55 %). Out of the minor defects, the most significant categories were the folded tail (34.24 %), normal isolated head (12.63 %), and coiled tail (5.81 %).

The differences identified in the seminal parameters, when contrasted with the age and weight of the animals, exhibited significant variations between the sampled groups, indicating an effect of these variables on plasma membrane and acrosome integrity, as well as DAB III mitochondrial function. DAB IV showed a significant correlation with the weight of the animal.

### *Sperm selection*

The efficiency of sperm selection using a discontinuous Percoll® gradient was compared with freshly ejaculated gamete. There is no significant difference between the two groups, as indicated in Table 3.

**Table 1**
Description of individual and semen collection data of *Leontopithecus chrysomelas* kept under human care.

| Group | Animal | Birth | Age (year) | Weight (g) | Semen collection (#) | Stimulation time (min) | pH | Ejaculate volume (µL) |
|---|---|---|---|---|---|---|---|---|
| **Old** | **1.a** | 2001/04/30 | 14 | 651.00 | 1 | 7.00 | 8.2 | 107.80 |
| | **2.a** | 2002/03/05 | 14 | 761.67 | 3 | 5.00 | 7,65 | 119.43 |
| | **3.a** | 2002/09/05 | 13 | 712.67 | 3 | 3.67 | 7.5 | 85.57 |
| | **4.a** | | 11† | 708.33 | 3 | 14.33 | 7.7 | 49.57 |
| | **5.a** | 2007/02/05 | 9 | 715.00 | 1 | 21.00 | 8.1 | 104.00 |
| | **6.a** | 2008/01/25 | 8 | 716.67 | 3 | 9.33 | 7.8 | 33.35 |
| **Group mean** | | | **11.5** | **710.89[a]** | - | **10.06** | **7.83** | **83.29** |
| **Young** | **1.b** | 2010/07/16 | 5 | 688.33 | 3 | 10.33 | 7.65 | 74.20 |
| | **2.b** | 2010/08/28 | 5 | 682.50 | 2 | 9.00 | 8.4 | 44.95 |
| | **3.b** | 2010/08/28 | 5 | 669.00 | 3 | 6.67 | 8.05 | 85.27 |
| | **4.b** | 2010/09/20 | 5 | 694.00 | 3 | 5.00 | 7.9 | 91.17 |
| | **5.b** | 2010/12/16 | 5 | 698.00 | 3 | 12.00 | 7.9 | 97.43 |
| | **6.b** | 2011/03/10 | 4 | 692.00 | 3 | 2.00 | | 45.30 |
| **Group mean** | | | **4.83** | **687.31[b]** | - | **7.5** | **7.98** | **73.05** |
| **p value** | | | | **0.0482238** | | **0.725995** | **0.465345** | **0.922687** |

The age was calculated at the time of collection. The data of weight, time, pH and ejaculate volume refers to the mean obtained from semen collections of each animal; †free-living animal (the age was estimated by the zoo). For each animal, three sample collections were expected; when the number is less than 3, it indicates failed collection attempts. [a, b] Different letters in the same column indicate a significant difference ($p< 0.05$) between ‘Old’ and ‘Young’ in the *Student’s t*-test.

**Table 2**
Seminal parameters evaluation and linear regression of *Leontopithecus chrysomelas* kept under human care.

| Parameter | Old (N=6) Mean ± SE | Young (N=6) Mean ± SE | ICC[1] | RM ANOVA[2] | P value | Regression | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Age (p value) | B | Weight (p value) | B |
| Concentration (x10$^6$sptz/mL) | 78.5×10$^6$ ± 15×10$^6$ | 97.2×10$^6$ ± 15.7×10$^6$ | 0.622 | 0.3054 | 0.1649 | 0.297 | -3.91×10$^6$ | 0.317 | -5.49×10$^6$ |
| Total motility (%) | 73.21 ± 5.94 | 76.00 ± 4.86 | 0.758 | 0.4878 | 0.0890 | 0.179 | -3.100 | 0.066 | -0.518 |
| Progressive motility (%) | 48.29 ± 8.13 | 47.20 ± 7.56 | 0.659 | 0.1855 | 0.9926 | 0.448 | -1.987 | 0.298 | -0.347 |
| Plasma Membrane Integrity (PMI) (%) | 86.04 ± 2.40$^a$ | 91.87 ± 1.48$^b$ | 0.931 | 0.0065 | **0.0496** | **0.021** | **-1.670** | **0.010** | **-0.231** |
| Acrosome Integrity (AI) (%) | 83.86 ± 1.90$^a$ | 91.80 ± 0.96$^b$ | 0.904 | 0.0319 | **0.0094** | **0.050** | **-1.256** | **0.009** | **-0.200** |
| DAB I (%) | 45.42 ± 5.18 | 54.21 ± 2.76 | 0.9308 | 0.0306 | 0.1918 | 0.055 | -2.547 | 0.063 | -0.343 |
| DAB II (%) | 26.42 ± 1.92 | 30.82 ± 2.65 | 0.5421 | 0.002 | 0.5391 | 0.224 | -0.457 | 0.080 | -0.082 |
| DAB III (%) | 17.58 ± 3.61 | 11.07 ± 1.52 | 0.8237 | 0.1597 | 0.0783 | **0.024** | **1.710** | **0.033** | **0.229** |
| DAB IV (%) | 12.54 ± 3.56$^a$ | 3.86 ± 0.66$^b$ | 0.9405 | 0.235 | **0.0267** | 0.077 | 1.746 | **0.016** | **0.275** |
| Morphology - major defects (%) | 21.75 ± 1.90 | 22.44 ± 2.86 | | | | 0.069 | 2.347 | 0.067 | 0.313 |
| Morphology - minor defects (%) | 62.00 ± 2.05 | 61.56 ± 3.84 | | | | 0.196 | -1.485 | 0.153 | -0204 |

Mean ± Standard Error. [1] ICC = Intraclass Correlation Coefficient, above 0.6 indicates consistency between replicates; [2] Repeated measures ANOVA, $p > 0.05$ indicates repeatability between replicates; [a, b] ($p < 0.05$), significant differences between "Old" and "Young" groups in the *Student t*-test. Regression: $p < 0.05$ indicates a significant correlation between the seminal parameters and the independent variables of Age and Weight (in bold). B is the non-standard regression coefficient. DAB I, high mitochondrial activity; DAB II, medium mitochondrial activity; DAB III, low mitochondrial activity; DAB IV, absence of mitochondrial activity. Abbreviation: DAB, 3,3' - diaminobenzidine.

**Table 3**
Liquid fraction seminal parameter comparison among pre and post sperm selection by discontinuous Percoll gradient.

| | Old (N=6) | | Young (N=6) | | ANOVA |
|---|---|---|---|---|---|
| | Pre-Percoll | Post-Percoll | Pre-Percoll | Post-Percoll | |
| Concentration (x10$^6$sptz/mL) | 78.5×10$^6$ ± 15×10$^6$ | 88.6×10$^6$ ± 21.1×10$^6$ | 97.2×10$^6$ ± 15.7×10$^6$ | 96.2×10$^6$± 17.0×10$^6$ | 0.106 |
| Total motility (%) | 73.21 ± 5.94 | 43.79 ± 9.682 | 76.00 ± 4.86 | 67.80 ± 7.41 | 0.462 |
| Progressive motility (%) | 48.29 ± 8.13 | 52.14 ± 8.735 | 47.20 ± 7.56 | 60.80 ± 8.62 | 0.540 |
| Plasma Membrane Integrity (PMI) (%) | 86.04 ± 2.40 | 83.50 ± 4.735 | 91.87 ± 1.48 | 91.40 ± 2.38 | 0.464 |
| Acrosome Integrity (AI) (%) | 83.86 ± 1.89 | 78.63 ± 6.85 | 91.80 ± 0.96 | 87.80 ± 3.25 | 0.151 |
| DAB I (%) | 45.42 ± 5.18 | 27.13 ± 11.94 | 54.21 ± 2.76 | 45.00 ± 1.17 | 0.281 |
| DABII (%) | 26.42 ± 1.92 | 20.38 ± 6.82 | 30.82 ± 2.65 | 42.80 ± 1.70 | 0.584 |
| DAB III (%) | 17.58 ± 3.61 | 15.25 ± 6.66 | 11.07 ± 1.52 | 8.80 ± 0.77 | 0.348 |
| DAB IV (%) | 12.54 ± 3.56 | 11.00 ± 5.70 | 3.857 ± 0.66 | 3.4 ± 0.86 | 0.634 |
| Morphology - major defects (%) | 21.75 ± 1.90 | 24.56 ± 5.07 | 22.44 ± 2.86 | 25.11 ± 2.91 | 0.941 |
| Morphology - minor defects (%) | 62.00 ± 2.05 | 50.44 ± 5.09 | 61.56 ± 3.84 | 54.44 ± 3.78 | 0.115 |

Mean ± Standard Error; ANOVA = Comparison between replica; $p<0.05$ indicates significant differences between samples pre and post sperm selection. DAB I, high mitochondrial activity; DAB II, medium mitochondrial activity; DAB III, low mitochondrial activity; DAB IV, absence of mitochondrial activity. Abbreviation: DAB, 3,3'- diaminobenzidine.

**Table 4**
Collection and RNA quantification data of golden-headed lion tamarin semen samples.

| | Pool (sample size) | | Volume (μL) | | Concentration (x10$^6$sptz/mL) | | Quantification Qubit HS RNA (ng) | | Quantification Qubit microRNA (ng) | |
|---|---|---|---|---|---|---|---|---|---|---|
| Animal | Seminal Coagulum | Liquid Fraction | Seminal Coagulum | Liquid Fraction | Seminal Coagulum† | Liquid Fraction | Seminal Coagulum | Liquid Fraction | Seminal Coagulum | Liquid Fraction |
| 1a | 1 | 1 | 89.5 | 60 | - | 3.69 | 0 | 0 | 0 | 0 |
| 2a | 3 | 3 | 736.8 | 27 | - | 45.2 | 145.6 | 0 | 147.2 | 142.6 |
| 3a | 3 | 3 | 378.9 | 270 | - | 65.37 | 64 | 0 | 290 | 196.4 |
| 4a | 3 | 3 | 302.7 | 310 | - | 50.13 | 71.2 | 70.4 | 246 | 294 |
| 5a | 1 | 1 | 104 | 40 | - | 11.23 | 99.2 | 0 | 69.4 | 0 |
| 6a | 3 | 3 | 257.6 | 265 | - | 63.05 | 77.6 | 77.6 | 126 | 502 |
| 1b | 3 | 3 | 225.6 | 180 | - | 39.2 | 135.2 | 0 | 199.6 | 73.6 |
| 2b | 2 | 2 | 196.9 | 150 | - | 40.95 | 0 | 61.6 | 0 | 388 |
| 3b | 3 | 3 | 525 | 260 | - | 79.74 | 0 | 80.8 | 94.4 | 438 |
| 4b | 3 | 3 | 402 | 300 | - | 116.19 | 111.2 | 52 | 168.2 | 0 |
| 5b | 3 | 3 | 298 | 440 | - | 109.08 | 0 | 79.2 | 60.8 | 290 |
| 6b | 1 | 1 | 63.1 | 150 | - | 1.44 | 142.4 | 65.6 | 77 | 0 |

†It was not possible to estimate sperm concentrations of seminal coagula.

*Spermatic RNA*

The number of collections and the volume obtained from semen samples pool (seminal coagulum and liquid fraction) for each animal are shown in Table 4. The pool average volume in the coagulated fraction was 298.34 μL (range 63–525 μL), but we were not able to estimate the concentration for these samples. The semen liquid fraction pool had an average volume of 224.58 μL (range 60–310 μL) with an average concentration of $52.10 \times 10^6$ spermatozoa/mL.

Table 4 also shows RNA quantification using HS (High Sensitivity) and microRNA Qubit kits. The average seminal coagulum RNA quantification was 70.53 ng (range 0–145.6 ng) and 123.2 ng (range 0–290 ng), respectively for HS and microRNA kits. RNA extraction from the liquid fraction produced an average of 40.6 ng (range 0–80.8 ng) and 193.7 ng (range 0–502 ng) of RNA, respectively for HS and microRNA kits.

The qualitative analysis of the samples evaluated in the Bioanalyzer showed a similar profile in both pools from the same individual (Fig. 2). The fluorescence peak emitted matches the microRNAs region and shows no differences between the profiles obtained by RNA from the seminal coagulum (Fig. 2-A) and the liquid fraction (Fig. 2-B). In both profiles we can observe the absence of peaks corresponding to the 18 S and 28 S subunits of ribosomal RNAs.

## Discussion

The establishment of genetic resource banks and the implementation of an assisted reproduction program for wildlife species can contribute to conservation strategies by facilitating genetic exchange between institutions and increasing the genetic variability of animals [39–41]. One of the first and essential steps in assisted reproduction is semen collection [42]. For *Leontopithecus chrysomelas*, we achieved a success rate of 86.1 % using the penile vibrostimulation (PVS) technique. PVS proves to be a consistent technique, as the results obtained here were similar to those in *Saimiri boliviensis* [43], *Callithrix jacchus* [22,44], and *Leontopithecus chrysomelas* [45,46]. Two instances of ejaculatory failure were observed in a single individual from the 'Old' age group, who exhibited an erection only in the final series of stimulation. Notably, a prior study [45] identified repeated failures in ejaculation in an elderly individual. Considering our comparable findings in an older subject, we cannot overlook the potential age-related association of this observation in older animals, although variations between individuals cannot be disregarded.

It is essential to consider that in the impossibility of reintroducing individuals into the wild, aging becomes a reality in *ex situ* management, and this factor can impact reproductive management. In addition to the observed failure in semen collection, in our study, we observed that older males were heavier than younger ones, and this increase in body mass was inversely correlated with seminal parameters. Despite the semen collection occurring in both the dry (April-September) and wet (October-March) seasons in São Paulo, Arakaki et al. [46] found no variation in body mass between these seasons in *Leontopithecus chrysomelas*. Similarly, while testicle size varied between seasons, seminal parameters did not show interference throughout the year in *L. chrysomelas* [46]. Although we did not measure the testicles, the same was observed in our study, where seminal parameters showed satisfactory intraclass correlation coefficients (above 0.6), supported by repeated measures ANOVA, indicating that the data are consistent with each other and that there was repeatability between the harvest replicas. Cui et al. [47] did not report similar findings, as they observed

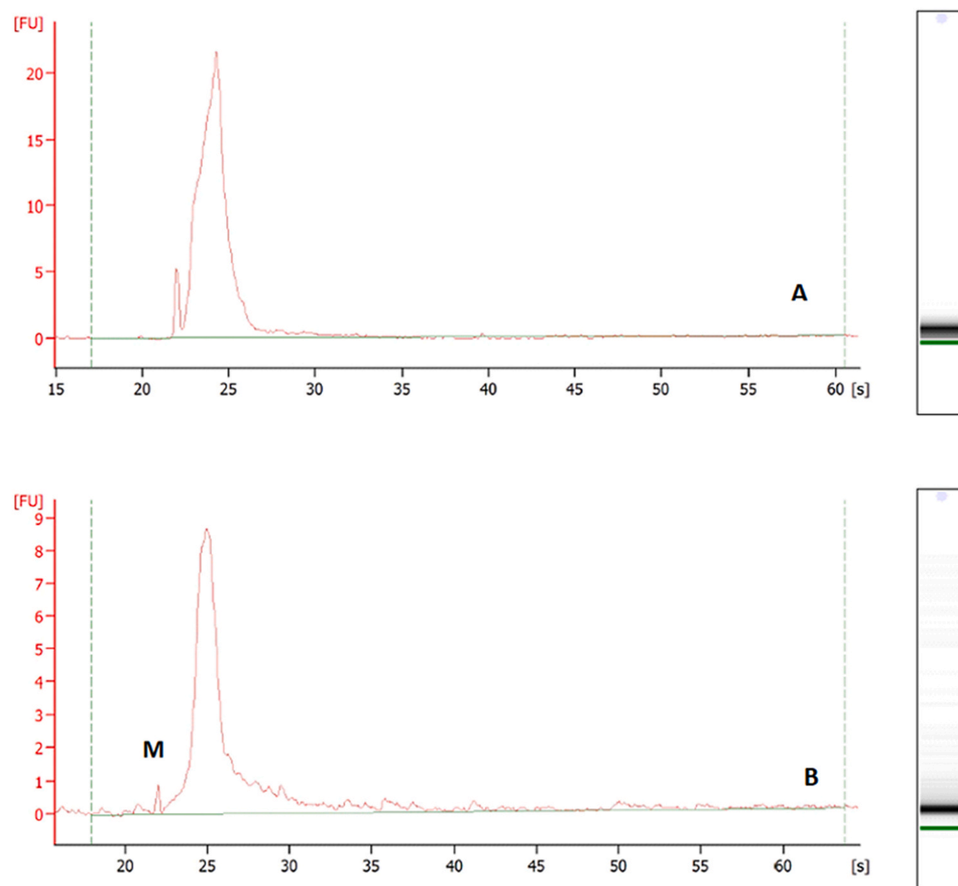


**Fig. 2.** Sperm RNA quality and amount profile measured. FU - Fluorescence Units; s- seconds; M - molecular marker (25 bp); A-seminal coagulum RNA sample; B-liquid fraction RNA sample.

significant intra- and inter-individual variations in ejaculates of *Callithrix jacchus,* possibly due to semen collection by electroejaculation, which demands different stimulus conditions, with the possibility of retrograde flow of the ejaculate and consequent loss of viable sperm. Conversely, our adoption of the PVS semen collection method seems to have ensured more consistent sampling. However, notable variations were observed between individuals, particularly when comparing different age groups. In these comparisons, older individuals exhibited lower scores of seminal parameters.

The aging process can be a crucial factor in male selection for reproduction [48], and our results reveal significant differences between the two sampled groups for the seminal parameters of plasma membrane integrity, acrosome integrity, and mitochondrial activity (Table 2). The first parameter is essential for the interaction of sperm with the epithelium of the female genital tract and for gamete fusion [17]; the second is crucial for the fertilizing potential of the sperm, as it refers to the vesicle containing enzymes essential for penetration of the zona pellucida [18]; and the assessment of mitochondrial function is relevant for sperm motility, homeostasis, and viability [19]. Despite reviews on the effects of age on reproductive decline in primates [49] and studies on cellular and biochemical modifications during the transition from puberty to adulthood in chimpanzees [27], few studies have been directed at neotropical primates for comparison [28]. However, marmosets exhibit a significant decline in testosterone levels in males from 6 through 15 years of age. [50]. In humans, compared to females, age exerts a more gradual influence on males, who rarely experience complete reproductive senescence [51,52]. However, an increase in the frequency of numerical and structural chromosomal alterations associated with age can be observed [53]. Indeed, changes in the male reproductive system can be attributed to decreased levels of circulating steroid hormones, such as testosterone and dehydroepiandrosterone (DHEA), which may lead to an increase in sperm abnormalities [49]. Our results contribute to this discussion, with the regressions showed in Table 2 indicating the impact (B) of age and weight on the reproductive potential of individuals. Each year of life represents a decrease of 1.67 % in plasma membrane integrity, 1.25 % in acrosome integrity, and an increase of 1.71 % in the observation of DAB III mitochondrial activity. The regressions also show that an increase in each unit of weight (g) results in a decrease of approximately 0.2 % in plasma membrane and acrosome integrity, while DAB III and IV mitochondrial activity would increase by 0.229 % and 0.275 %, respectively. In summary, our results reveal the association of older and heavier males with lower scores for some seminal parameters, specifically concerning plasma membrane and acrosomal integrity, as well as mitochondrial activity. In *Callithrix jacchus*, a positive correlation between male weight and the incidence of sperm morphological abnormalities was also observed [31]. These results have implications for the quality of reproductive parameters of animals available for captive breeding, including the application of reproductive biotechnologies. It is important to consider this when selecting males for reproductive management, as well as for the use of their semen in reproductive biotechnologies. The maintenance of genetic resource banks can minimize problems associated with senescence and the availability of individuals for reproduction, especially through the inclusion of genetic material from free-ranging animals or those from other institutions. The results of this study underscore the importance of cryopreserving samples while individuals are young, as representative samples of good quality are required for gamete cryopreservation [12]. However, this does not preclude the use of older animals; rather, it underscores the benefits of collecting samples from them during their youth.

The preservation of semen in genetic banks results in a loss of sperm viability due to damage from freezing and thawing, including acrosome rupture, damage to the plasma membrane, and loss of motility [54]. Sperm selection techniques are crucial for mitigating these effects and facilitating the removal of infectious agents, dilution media, and cryoprotectants from samples designated for reproduction [33]. One such method is the discontinuous Percoll® gradient, which, akin to other protocols in reproductive biotechnology, requires standardization for the selection of viable gametes from *Leontopithecus chrysomelas*. Previous studies have shown that Percoll® has no harmful effects compared to *Pure Sperm*®, indicating cost-benefit advantages [55,56]. However, despite selecting gametes, the use of the Percoll® gradient, performed only in the liquid fraction of the ejaculate, did not achieve the expected efficacy in *Leontopithecus chrysomelas*, especially in individuals from the 'Old' group, for whom the decrease in semen quality parameters was more evident (Table 3). Although the changes were not significant, the results emphasize age as a factor to be considered in the application of biotechniques.

The coagulation of semen poses a challenge for utilizing gametes from some Platyrrhini primates in reproductive biotechniques [21]. Various techniques have been proposed for coagulum dissolution, such as the use of proteolytic enzymes and diluents based on coconut water in many species [57–62]. The presence of coagulum poses a problem in species that produce coagulated semen, and the absence of treatments for it results in the loss of viable gametes, as this coagulum is typically discarded after separating the liquid fraction [26]. The use of coagulum in biotechniques has been feasible in *Macaca mulatta*, involving the liquefaction of a portion of the clot to release spermatozoa for use [63]. Similar efforts have been made in neotropical primates [8,64]. Consequently, the spermatozoa contained in the seminal coagulum, which does not liquefy, constitute a rich source of genetic material for researching the genetic foundations of reproduction, including factors associated with fertility and genetic disorders. RNA profiles can serve as markers of seminal quality, characterizing a non-invasive tool for assessing male fertility in cattle [65]. The population of small spermatic RNAs can also impact male fertility, suggesting an indirect proportional relationship between the concentration of these transcripts and fertility [66]. The significant challenge in using spermatozoa contained in the coagulum is isolating the gametic cell from somatic cells. One way to verify samples of spermatozoa free from somatic cell presence is through the absence of ribosomal RNA subunits [67–69], as during spermiogenesis, gametes lose most of the cytoplasmic content, including the majority of RNAs. The RNA extraction protocol used here indicated lower concentrations of larger RNA molecules, with the majority of spermatic RNAs represented by small molecules. In the quantification evaluated on the Bioanalyzer (Fig. 2), peaks corresponding to ribosomal RNAs are absent, indicating that somatic cells were removed with the discontinuous density gradient selection and the protocol described in the methodology, adapted from Rauber [37]. The findings could enhance the semen collection effectiveness for tamarins, where the liquid fraction (extender plus sperm cells) can be used in reproductive biotechniques, while the fraction of spermatozoa contained in the coagulum can be utilized, for example, as a non-invasive approach to evaluating the fertility of golden-headed lion tamarins and other closely related Neotropical primates. The complexity of transcripts present in spermatozoa has sparked interest in identifying RNAs, whether messenger or non-coding, delivered during fertilization and their respective functional role in embryo activation and early embryo development [69,70]. The extraction protocol is the first step in investigating the role of spermatic RNAs as epigenetic mediators in Neotropical primates, i.e., their contribution during fertilization and their respective functional role in embryo activation and early development stages.

## Conclusion

The impact of age and weight on seminal parameters, as highlighted in this study, can guide decisions related to the reproductive management of the golden-headed lion tamarin and other closely related species. Whether for the selection of partners in natural reproduction or the implementation of assisted reproduction techniques, these findings underscore the importance of cryopreserving semen from males at a young

age to safeguard the reproductive potential of individuals. This is particularly crucial when combining high-quality samples with effective techniques for selecting viable sperm post-thawing. Moreover, the study emphasizes the valuable role of utilizing the seminal coagulum in molecular investigations. With straightforward adjustments, this often-discarded material yielded results comparable to those obtained by extracting RNA from the liquid fraction of semen. This enhances the semen utilization potential, with its liquid fraction applicable to reproductive biotechnologies, while the spermatozoa within the clot can be employed in non-invasive fertility assessment approaches. In the context of wild species, especially those facing some degree of threat, optimizing the use of biological samples becomes imperative, given their precious nature and the considerable effort involved in their collection. The utilization of this material in molecular studies serves as a meaningful avenue when its application in assisted reproduction techniques is unfeasible. Furthermore, the validation of the RNA extraction protocol for the species lays the groundwork for exploring the contribution of the male gamete and the factors influencing reproductive success—considerations integral to the development of reproductive biotechniques.


## Funding

This work was grant from the São Paulo State Foundation for Research Support (FAPESP: 13/22339–0) to ACP.


## CRediT authorship contribution statement

**Andrea Cristina Peripato:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Rodrigo del Rio do Valle:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Bruno Sauce:** Writing – review & editing, Supervision, Investigation, Formal analysis, Data curation. **Paloma Rocha Arakaki:** Writing – review & editing, Investigation, Data curation. **Isabela Midori Watanabe:** Writing – original draft, Investigation. **Patrícia Hergert Bacher:** Writing – original draft, Validation, Project administration, Methodology, Investigation, Formal analysis, Conceptualization.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.


## Acknowledgments

We thank to the technical team of the São Paulo Zoo Foundation, especially to Paula Andrea Borges Salgado, for assistance in sample collection and to Professor Dr. Reinaldo Alves de Brito, for the laboratory support. PHB and IMW were recipients of Fundação Parque Zoológico de São Paulo master's fellowship, Programa de Pós-graduação em Conservação da Fauna/UFSCar.


## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.therwi.2024.100098.

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